Switchable Biosensor Devices

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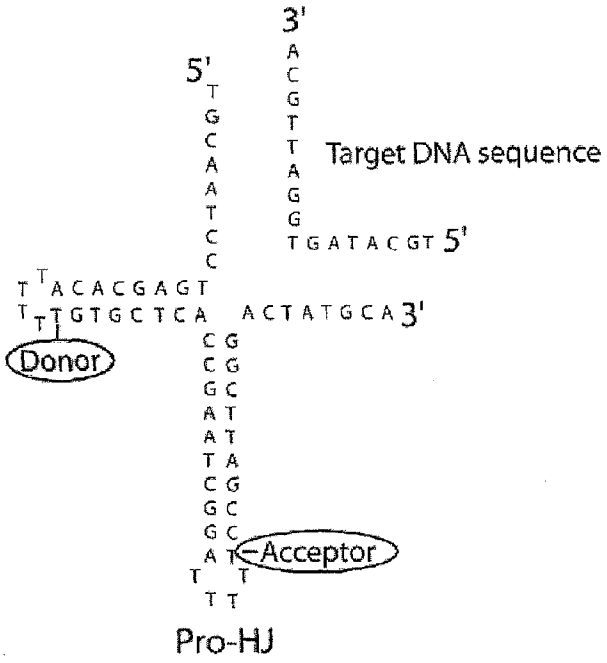

(57) Abstract: The present invention provides a method for detecting a target analyte in a sample, using a pro-macromolecular structure switch which has an analyte binding site for binding the analyte to form together, a macromolecular switch, with an ion coordination site, and arms flippable between discretely different conformations corresponding to different ion binding conditions. The different conformations provide, with an output signal reading system, characteristic output signals. The pro-switch is contacted with sample and an external input signal applied to the input device to change ion binding conditions to flip switch conformations, and changes in output signal are read out. The invention also provides a pro-macromolecular structure switch.

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SWITCHABLE BIOSENSOR DEVICES

The present invention relates to the use of novel switchable macromolecular structures for specific analyte detection.

There is a substantial need for detecting various biomolecule analytes, especially DNA and RNA sequences, with high specificity and high sensitivity, and a wide range of procedures has been developed for meeting this challenge. In general though, currently available procedures suffer from various disadvantages such as the need for labelling of analyte material, and the need for precise measurement which result in the need for more or less complex multi-step processing by the user, which in turn increases the risk of variability and formation of artefacts etc, at each additional processing step. Another particular problem is that of false positives resulting from non-specific binding to the probe and/or substrate, by other moieties in the test sample similar to the analyte.

DNA hybridisation assays are fundamental to much of life sciences research and especially in the application of DNA microarrays. The generation of such arrays allows the simultaneous measurement of the expression of tens of thousands of the corresponding messenger RNA molecules. Measurement of RNA molecules is generally achieved in an indirect manner by transformation to DNA via the multistage enzymatic process of reverse transcription. During this process, a certain proportion of fluorescently labelled nucleotide is introduced into the DNA molecule. When these fluorescently labelled DNA targets are hybridised with the array of DNA probe molecules, binding of target to probe molecules can be detected using confocal fluorescent scanning. The process of reverse transcription and labelling is, however, known to introduce variability into microarray experiments and can be a source of error since mRNA sequences in the extracted mixture may not be represented in the same proportion in the final labelled DNA mixture. Gupta V al J. *Nucleic Acids Res.* **2003**, *31*, 13, Baugh, L.Ret al. *Nucleic Acids Res.* **2001**, *29*, 29 Schuchhardt, J et al. *Nucleic Acids Res.* **2000**, *28*, 47

In addition, the requirement of reverse transcription makes the process of microarray experiments significantly more expensive in terms of time, consumables and necessary equipment and is a barrier to the application of microarrays in (for example) a diagnostic setting.

Label-free methods of detection that have been applied to this problem, include the use of electrochemistry. For example, a label-free method has been developed which exploits the relatively low oxidation potential of guanine. By designing probes in which guanine has been substituted with inosine (which does not oxidise at such a low potential but which forms base pairs with cytosine), hybridisation will give rise to an oxidation current Thorpe, H.H. *TRIBTECH* 1998, 16, 117-121 Leiber and co-workers have also published a method of detecting hybridisation at silicon nanowires, using PNA probes, on the basis that hybridisation will lead to a change in conductance Hahm, J-I, Lieber, C.M. *Nano Lett.* **2003.** DNA microarray technology demands a high degree of multiplexation (current arrays available from Affymetrix have 1.3 million oligonucleotides) and the technology required to read such an array electrochemically is currently not available.

Our earlier Patent Application WO2004/099767, discloses inter alia a macromolecular switch suitable for use in a data acquisition and/or processing device, comprising: a macromolecular structure having at least one ion binding site, and flippable between a plurality of discretely different conformations corresponding to different ion binding conditions at said at least one ion binding site, at least in the absence of any other binding to said macromolecular structure, said macromolecular structure being provided with at least one electrochemical input device formed and arranged for applying such different ion binding conditions to said ion binding site in response to corresponding external input signals. This publication further discloses the possibility of specific label-free analyte detection, by utilizing macromolecular structures which incorporate moieties which can be selectively displaced and replaced by the target analyte, by means of competitive binding. Reliance on competitive binding, is however, generally less desirable, because of the difficulty of developing specific, robust assays of this type.

Thus there remains a need for label-free analyte detection, which minimizes the demands on the user. There is also a need for enhanced specificity and sensitivity in analyte detection.

It is an object of the present invention to avoid or minimize one or more of the abovementioned disadvantages.

We have now found that it is possible to detect biomolecular analytes such as target DNA and RNA sequences, with high specificity and high sensitivity, without the need for analyte labelling, by means of the use of a pro-macromolecular structure switch which binds together with a target analyte, so as to form a functional macromolecular structure switch such as those described in our earlier WO2004/099767. This in turn enables signalling of the detection of target analyte in a particularly simple and convenient manner. As discussed in WO2004/099767 and further discussed hereinbelow, a particularly preferred form of macromolecular structure switch is based on the Holliday Junction. The DNA Holliday Junction structure is a junction of four double helices first reported in 1964 Holliday, R. *Genet. Res.* **1964,** *5*, 282. The unique topological element of the junction is a branch point discontinuity formed at the intersection of the component strands. The overall structure of the open junction is determined primarily by the strong electrostatic repulsion between the phosphate groups. In low ionic strength solutions, this repulsive Coulombic interaction favors an extended structure determined by maximum charge separation, an open branch point region and approximate fourfold symmetry.

The addition of certain cations which bind to a selective ion binding site at the branch point, at a concentration above a critical concentration level, leads to screening of the inter-phosphate repulsion and induces a conformational transition to a more compact, folded junction. Here the branch point collapses to enable pairs of double helical arms to align coaxially and cross in the so-called stacked-X conformation. Ortiz-Lombardia, et al *Nature Struct.Biol.* **1999,** *6*, 913 Each stacked duplex has one non-exchanging strand running along the exterior of the junction and one sharply bent interior exchanging strand. Therefore a 4-way junction can form one of at least two alternative stacking conformations (Hays et al., *J. Biol Chem* **2003** 278, 49663).. The Open and Closed structures are shown schematically in Figs 17A and 17B, respectively.

The principle of using a Holliday Junction (HJ) as a sensor is based on the use of a pro-Holliday Junction (pro-HJ). On its own the pro-HJ adopts an informal folded structure but on hybridization with a target oligonucleotide analyte forms a complete HJ (see Figure 1 which shows schematically a target analyte about to hybridize with a pro-HJ so as to form a corresponding HJ). Hybridization of the pro-HJ with a target oligonucleotide forms a switchable HJ, whose conformation can be probed using various methods without the need for labels on the target oligonucleotide. Only when this Pro-HJ hybridises with the target

oligonucleotide sequence analyte, is a functioning switch formed. Switching occurs between differerent (Open and Closed) conformations of the branch-point which is controlled by ionic binding and can be detected – for example by FRET. The formation and functional switching of the HJ branch-point upon binding of the specific target sequence provides a stringent control of specificity assuring against non-specific interactions. We have found that such a nanoscale switch can act as a specific sensor for either complementary DNA or RNA, with high sensitivity. Thus the switchability of the HJ is used as proof that a specific hybridization has occurred. In contrast to molecular beacons, such hybridization does not require the breaking of secondary structure in the probe molecule and there are therefore no intrinsic energetic barriers to be surmounted.

Thus in a first aspect the present invention provides a pro-macromolecular structure switch suitable for use in detecting an analyte, wherein said pro-switch has an analyte binding site formed and arranged for binding said analyte with high specificity so as to form together therewith,
a macromolecular structure switch
having at least one ion binding site, and
flippable between a plurality of discretely
different conformations
corresponding to different ion binding conditions
at said at least one ion binding site,
at least in the absence of any other binding to said macromolecular structure,
wherein said discretely different conformations are capable of
providing, in use, with an output signal reading system, characteristic output signals and wherein said pro-macromolecular structure switch is substantially incapable of being flippable between said plurality of discretely different conformations and providing, in use, with said output signal reading system, said characteristic output signals.

The present invention also provides a pro-macromolecular structure switch suitable for use in detecting an analyte, wherein said pro-switch has an analyte binding site formed and arranged for binding said analyte with high specificity so as to form together therewith, a macromolecular switch, comprising: an oligomeric macromolecular structure having at least one selective ion coordination site, and arms flippable between a plurality of narrowly defined discretely different conformations of said oligomeric macromolecular structure

corresponding to different ion binding conditions at said at least one selective ion coordination site, at least in the absence of any other binding to said oligomeric macromolecular structure which would interfere with flipping between said different conformations in response to a change between said different ion binding conditions, wherein said discretely different conformations are capable of providing, in use, with an output signal reading system, characteristic output signals, and wherein said pro-macromolecular structure switch has a substantially different switching functionality from that of said oligomeric macromolecular structure and is substantially incapable of providing, in use, with said output signal reading system, said characteristic output signals.

It is important to note that the macromolecular structures used in accordance with the present invention are flippable between more or less well defined discretely different conformations or structural forms of a bound set, i.e. a finite limited predetermined number, of different conformations. This is quite different from previously known systems in which it has been proposed to use macromolecules which are described as being switchable between different "states", which typically correspond to one state in which the macromolecule is bound to some other moiety, and another state in which it is not bound to said moiety, and/or involve one or more conformational states which is (are) not well defined to a greater or lesser extent and/or are part of a large spectrum or continuum of different conformations, and/or actually involve dissociation into multiple separate macromolecular structure entities. The use of the particular type of flippable macromolecular structures involving a flipping, which has a generally mechanical character, of a single macromolecular structure entity, according to the present invention, provides better defined and quantized or digitized switching characteristics with more clearly and positively defined switch states which leads to significant benefits such as more reliable and reproducible operation.

For the avoidance of doubt, it should be noted that the following terminology used herein in relation to various features of the present invention is intended to encompass the following meanings, unless otherwise specifically indicated.

The qualifier "pro-" is used herein in accordance with common practice in the field of biotechnology, to indicate a precursor which is converted, in use of the precursor, to the entity being qualified. Thus in the present case this term indicates a precursor with a

"switching" functionality that differs significantly from that of the oligomeric macromolecular structure switch.

The term "detecting" indicates qualitative and/or quantitative detection, including inter alia simply detecting the presence or absence of a target analyte, and simply detecting a change in the concentration of a target analyte.

The expression "oligomeric macromolecular structure" indicates a macromolecular entity of intermediate relative mass. In general this corresponds to a structure consisting essentially of a switch device, including any associated components such as anchoring and/or switch state signaling component(s), but substantially free of any other non-functional components. Typically the oligomeric macromolecular structures of the invention will comprise not more than 500, preferably not more than 250, most preferably not more than 150, repeating units such as nucleotides, amino acids, saccharides.

The expression "selective ion coordination site" indicates a site which can selectively bind certain ions. In certain preferred forms of the invention, said site may comprise an "ion coordination pocket". The expression "ion coordination pocket" indicates a structural feature which contributes energetically and/or spatially to the selectivity of the binding of ions thereto.

The expression "ion binding conditions" includes physico-chemical conditions such as concentration, ionic strength, temperature, electric field strength etc, the effect of differences in the structure of the macromolecular structure switch such as those due to the specific sequence at the HJ branch point and also features such as the nature, identity, ion size, etc of the particular ion species involved in binding to the ion binding site(s).

In another aspect the present invention provides a method for detecting a target analyte in a sample, which method includes the steps of:

providing a pro-macromolecular structure switch,

wherein said pro-switch has an analyte binding site formed and arranged for binding said analyte with high specificity so as to form together therewith,

a macromolecular structure switch

having at least one ion binding site, and

flippable between a plurality of discretely
different conformations
corresponding to different ion binding conditions
at said at least one ion binding site,
at least in the absence of any other binding to said macromolecular structure,
wherein said discretely different conformations are capable of
providing, in use, with an output signal reading system, different characteristic output signals
and wherein said pro-macromolecular structure switch is substantially incapable of being
flippable between said plurality of discretely different conformations and providing, in use,
with said output signal reading system, said different characteristic output signals;
contacting said pro-switch with a said sample under binding conditions, so as to form a said
macromolecular structure switch with any said target analyte present;
providing a said output signal reading system;
applying an external input signal to said at least one input device so as to change the ion
binding conditions at said ion binding site so that said macromolecular structure switch is
flipped from a first said conformation to a second said conformation; and
reading out any change in said output signal.

The present invention also provides a method for detecting a target analyte in a sample,
which method includes the steps of: providing a pro-macromolecular structure switch
wherein said pro-switch has an analyte binding site formed and arranged for binding said
analyte with high specificity so as to form together therewith, a macromolecular switch,
comprising: an oligomeric macromolecular structure having at least one selective ion
coordination site, and arms flippable between a plurality of narrowly defined discretely
different conformations of said oligomeric macromolecular structure corresponding to
different ion binding conditions at said at least one selective ion coordination site, at least in
the absence of any other binding to said oligomeric macromolecular structure which would
interfere with flipping between said different conformations in response to a change between
said different ion binding conditions, wherein said discretely different conformations are
capable of providing, in use, with an output signal reading system, characteristic output
signals, and wherein said pro-macromolecular structure switch has a substantially different
switching functionality from that of said oligomeric macromolecular structure and is
substantially incapable of providing, in use, with said output signal reading system, said
characteristic output signals;

contacting said pro-switch with a said sample under binding conditions, so as to form a said macromolecular structure switch with any said target analyte present;
providing a said output signal reading system;
applying an external input signal to said at least one input device so as to change the ion binding conditions at said ion binding site so that said macromolecular structure switch is flipped from a first said conformation to a second said conformation; and
reading out any change in said output signal.

For the avoidance of doubt, the term "biomolecule" is used herein to encompass not only molecules found in living organisms, but any molecule having a character substantially similar to such molecules, howsoever, obtained. Thus, for example, biomolecule includes oligonucleotides or polypeptides whether made by processes having a biological character or purely organic chemistry synthetic processes, and oligonucleotides or polypeptides which are entirely artificial, i.e. are not found in nature.

It should also be noted for the avoidance of doubt that references to "macromolecular structure" herein, include both discrete individual molecules and macromolecular structure entities comprising more or less closely bound combinations of a plurality of discrete individual molecules, the integrity of which combinations is maintained across said discretely different conformations . Thus, for example, a Holliday Junction (HJ) (discussed in more detail elsewhere herein) is generally a combination of four nucleic acid strand sequences (or like entities) bound together into a single macromolecule entity with four arms, each said arm comprising respective portions of two strand sequences bound to each other. It will be appreciated that these four sequences may all be part of separate strands, or two or more of said sequences may be part of a single strand with a molecular linkage extending between them, said strand being suitably folded to allow said sequences to hybridize with other sequence portions so as to form the HJ (or pro-HJ) structure. One type of molecular linkage will use co-valent cross-linking between strands. In another approach, molecular linkages comprise strand sections which link the ends of strand sections which are hybridized together to form an arm of the pro-HJ. Such linkages generally comprise any neutral sequence of nucleic acid bases which would not interfere with binding between complementary strand sequences to form the arms of the desired HJ structure, such as e.g. TTTT see Figure 1. Advantageously, a pro-HJ macromolecular structure switch comprises a single polynucleotide strand including six said strand sequence portions, wherein four said

strand sequence portions are hybridized together so as to form two HJ arms (double-stranded), and the other two said strand sequence portions form two HJ pro-arms (single-stranded), which together provide said target analyte binding site, and when having target analyte bound thereto, constitute the other two arms of the HJ macromolecular structure switch.

It will be appreciated that the total output signal obtainable from a switch device of the present invention will depend on the number or population size of individual pro-macromolecule structures used in each device. Preferably therefore the device of the present invention has a plurality of said macromolecule structures. Desirably there is used at least 5, more desirably at least 10, preferably at least 100, advantageously at least 1000, most conveniently at least 10,000, pro-macromolecule structures, in each switch device.

It will be appreciated that the pro-macromolecular structure should generally be formed and arranged to facilitate applying the different ion binding conditions to said ion binding site of the macromolecular structure formed upon binding of the pro-macromolecular structure with target analyte. In general this involves retaining the pro-macromolecular structure in proximity to said input device, in a "fluid-interfaceable condition". As used herein, the expression "fluid – interfaceable condition" means that the pro-macromolecular structure is supported in any convenient manner which would permit interaction between the macromolecular structure and a fluid medium. Conveniently the pro-macromolecular structure may be anchored in a suitable manner to a substrate having a suitable form, so that said structure can interact with components present in a fluid medium with which the device is contacted in use of the device. Thus whilst the structure would generally be mounted on the surface of a solid substrate, it could also be embedded inside a more or less permeable substrate, which may moreover be more or less flexible, such as, for example a gel. Naturally in such a case the gel should be chosen as to have a pore size sufficiently large to enable the target components in the fluid medium to be able to penetrate the gel and come into contact with the structure. It is also important that the gel pore size should be sufficient to allow adequate freedom of movement of the structure between its first and second conformations. Furthermore the pro-macromolecular structure could also in principle simply be suspended in solution within a cell or other vessel provided with said input device. Such a cell may moreover be defined by fluid impermeable walls, or in some cases, at least partly, by fluid permeable walls such as dialysis membrane. By suitable choice of the pore size of

such a membrane it is moreover possible to entrap the pro-macromolecular structure within a zone in direct contact with the electrochemical input device, with a sample solution containing target biomolecule analyte being contacted with the outside of the membrane.

It will of course also be appreciated that at least some macromolecular structures, especially biomolecular ones, may be susceptible to denaturation or other damage to a greater or lesser degree under certain conditions e.g. high and/or low pH, and it is therefore desirable that this should be taken into account in relation to the properties of any medium through which the pro-macromolecular structure is supported and/or interfaced through with biomolecule analytes. Alternatively or additionally, pro-macromolecular structures may be modified so as to stabilize them to a greater or lesser extent against denaturation or disintegration, for example by means of co-valent and non-covalent cross-linking of the hybridized polynucleotide sequences within one or more arms of an HJ. Examples of covalent cross linking includes psoralen, (Saffran et al. *Proc. Natl. Acad. Sci.* **1982**, 79, 4594-8) and Iodo dT / phosphotioate modification (Roche, C.J. and Tse-Dinh, Y.C. *Int. J. Biol. Macromol.* **2001**, 29, 175-180). . Examples of non-covalent cross linking include triple helix structures (Welch et al., *Nucleic Acids Res.* **1993**, 21, 4548-4555) and hairpin polyamides (Herman, D.M.; Baird, E.E.; Dervan, P.B. J. Am. Chem. Soc. 1998, 120, 1382-139. An example pro-HJ using cross linking is illustrated in Figure 2.

Alternatively macromolecular structures may be stabilized by increasing the number of bonds. For example it is known that short armed HJ structures disassemble in low salt conditions (Shida, T. et al. J. Biochem. 1986, 119, 653-658)) but longer-armed structures are more stable. An additional benefit of the use of bridging sequences e.g. TTTT at the end of the HJ arms to form uni-, bi- or tri- HJs or pro-HJs, as described above, is that this would also increase the stability of the HJ structures against strand disassociation.

This can be useful where it is, for example, desired to remove biomolecules which have been bound to biomolecule analyte binding sites on an HJ, without loss of the HJ structure in order that it can continue to be used. More stable HJ structures are also obtainable by means of using synthetic polynucleotide analogues based on the use of size-expanded analogues of the natural bases (for example, using a three ring analogue of a bicyclic purine and a bicyclic analogue of a monocyclic pyrimidine as discussed by Liu et al in Science, Vol 302 Issue 5646, 868-871 (2003). (For the avoidance of doubt, unless the context specifically requires

otherwise, the term HJ is intended to encompass modified HJs.) Increased stability may also be achieved to a greater or lesser degree by the use of pro-HJs wherein the number of separate strands (as discussed hereinbefore) is minimized.

Modifications to the oligonucleotide(s) of oligomeric macromolecular structures of the present invention may also be used to modify the sensitivity of target analyte detection. Thus for example, locked nucleic acid (LNA) modifications (wherein the ribose moiety of the LNA nucleotide is modified with an extra bridge connecting 2' and 4' carbons which "locks" the ribose in a 3'-endo structural conformation),
may be incorporated into the pro-HJ. The effect is dependent on the position within the pro-HJ with modifications remote from the branch point being preferred. Other possible modifications which may be mentioned in this connection include, non-natural bases such as size-expanded analogs of nucleotides (Liu et al., Science Vol 302 pg 268-871), and other like chemical alterations to the poltnucleotide backbone or base structure.

Nucleic acid modifications may also be used to enhance the switching performance of oligonucleotides macromolecular switches. Possible modifications which may be mentioned in this connection include LNA, PNA, phorphorthioates, non-natural bases such as size-expanded analogs of nucleotides, and other like chemical alterations to the polynucleotide backbone or base structure.

In embodiments where the pro-HJ is anchored or tethered to an electrode surface, several factors should be borne in mind. The electrode can be chosen from any of an assortment of conductors or semiconductors. These range from metals such as silver, gold, platinum, titanium, tantalum, tungsten, aluminium, nickel, zinc, copper and alloys of these or other metals to semiconducting or conducting oxides, nitrides or mixed oxynitrides of metals such as titanium, nickel, iron, tin, indium, tantalum, strontium, iron, tungsten, niobium, iridium, molybdenum, hafnium, zinc, aluminium and zirconium and doped versions thereof. Electrodes may also be chosen from materials such as carbon, conducting and semiconducting molecular organic systems, inorganic and organic charge transfer salts (e.g. tetrathiafulvalene-tetracyanoquinodimethane), and semiconducting materials such as silicon, gallium arsenide or conducting polymers such as polypyrrole, polythiophene or polyaniline.

In accordance with the present invention, a biomolecule may generally be anchored to an electrode or other suitable substrate using one or more of three principal approaches: Physisorption, chemisorption or covalent attachment.

Physisorption is procedurally relatively simple and relies on non specific interaction between areas of, for example, complementary charge or similar hydrophobicity. It tends not to impart controlled orientation and may be reversible under conditions of varying pH, ionic strength or solvent hydrophobicity (Logen et al., Environ. Sci. Technol. 2005, 39, 6371-6377)

Chemisorption is a more specific form of orientation since it relies on the favoured interaction between a surface and a particular moiety on a molecule, examples of such are the interaction between thiols and gold (Whitesides et al., Chem. Rev. 2005 105, 1103-1169) and the interaction between phosphonic acids and metal oxides (Long B., Nikitin K., Fitzmaurice D., J. Am. Chem. Soc. 2003, 125, 5152-5160).

Covalent attachment of biomolecules relies on the modification of an electrode surface so that it is intrinsically active to or can be activated to attach to specific, functional groups on the biomolecule. Suitable treatment might be the oxidation of carbon electrodes to form carboxylic groups on the surface which can be activated to bind with amine groups Campbell et al., Langmuir 2001 17, 3667.. Another example would be the use of a functional silane compound (for example an epoxide terminated silane) to form a self-assembled, covalently attached layer that can be used to covalently attach a polymer on, for example, a metal oxide Cass, Anal. Chem 2001 73, 2676-2683. Specifically, epoxysilane can be polymerised onto a metal oxide such as titanium dioxide to form a surface with active epoxy groups that can react with amine functions on the surface of a biomolecule. In both such cases, the surface coating can be made sufficiently thin so as to not hinder electron transfer across the interface. Thus an amino group on the 5'-terminal of one arm of the HJ structure or an internal NH_2 dT linkage may itself be used for covalent bonding directly to a substrate. Alternatively a linking moiety such as a biotin molecule may be attached to the 5'-terminal of one arm of the HJ structure. Although it is generally more convenient to use the 5'-terminal of arm III of the HJ structure for anchoring, it is nevertheless also possible to effect anchoring via the 3'-terminal if desired.

Such techniques can moreover be combined to form a suitable surface for biomolecule anchoring. Thus, for example, a polyion such as protonated poly-l-lysine can be physisorbed onto a charged electrode surface on the basis of charge pairing. As the poly-l-lysine is a polypeptide with amino side chains, the amino groups can be attached to activated carbonyls on a biomolecule or cross linked to a biomolecule in order to give covalent attachment. Chemisorption of a bifunctional alkyl thiol can convert a gold surface into a surface reactive to biomolecular binding. For example mercaptopropionic acid can be chemisorbed onto gold to give a self-assembled monolayer with carboxylic acid moieties reaching into the bulk solution. These carboxylic groups can be activated by carbodiimide chemistry to react with amine groups on a biomolecule.

The pro-switches can also be anchored by means of physical entrapment. This involves the formation of a polymer network or sol-gel or hydrogel substrate that has a pore size dimensioned so as to enable it to entrap a biomolecule during its formation. Although this can give a stable environment for the biomolecule, the surrounding matrix limits its interaction with other biomolecules to a greater or lesser degree. Also, since entrapment is not a covalent immobilisation it may be prone to leaching to a greater or lesser degree. Accordingly it is important to ensure that when a gel or other polymer network substrate is used, this has a suitable pore size and suitable cavity size for effectively retaining the structure within the substrate whilst allowing it sufficient freedom of movement between its first and second conformations, as well as allowing freedom of access of target biomolecules to the entrapped biomolecule structure. Suitable gels include agarose, polyacrylamide gels, polysaccharide hydrogels, for example carboxy-methyl dextran. It will be appreciated that when gels are used, the pro-macromolecule structures can also be covalently bonded to the gel matrix in order to prevent escape thereof over a period of time. Furthermore, pro-macromolecule structures can also be "anchored" by means of relatively strong non-covalent bonding e.g. biotin binding to streptavidin.

The pro-macromolecular structure switches of the invention may moreover be incorporated in a wide variety of different kinds of apparatus adapted for use in various types of in vitro assay system. Thus, for example, the pro-switches may be incorporated in eppendorf tubes, multi-well plates, or in gels, or single channel analysis devices in conventional or micro-scale systems. Example configurations are illustrated in Figures 7-12.

In one arrangement, the switchable biosensors could be used in a industry standard multiwell plate format, containing typically 96, 384 or 1536 wells, or alternatively in a bespoke plate of multiple wells of the same or different sizes, with a population of pro-macromolecular switches deposited in each well, either in solution or with the pro-macromolecular switches immobilized to the inside of the well. Wells would each typically contain distinct homogeneous populations of pro-macromolecular switches, with solutions of test sample added, but could include replicate assays or assays based on heterogeneous pro-macromolecular switch populations. In this configuration an optical separation-sensitive output device such as FRET could be read using a standard fluorescence plate reader, typically based on a cooled CCD camera, reading fluorescence intensity or fluorescence lifetime measurements. This arrangement is illustrated in Figure 7. In other configurations, optical signals could be detected in optical fibres positioned into each microwell, or electrochemical signals could be detected using electrodes configured within the wells. Ionic switching of the switchable biosensors could be achieved by introduction of ionic solutions into each well using fluid-handling robotics, or electrochemically generated using electrodes configured within each well. In a further variation, pro macromolecular structures could be attached onto microbeads, desirably of 1-70 μm, which are arranged in micro-scale wells of different or similar size as illustrated in Figure 9.

In another arrangement, the switchable biosensors could be used in a microarray format, where a population of pro-macromolecular switches would be immobilized into micro-scale features or spots, with pro-macromolecular switches immobilized on a solid substrate or within a gel matrix as decribed hereinbefore. Spots would each typically contain distinct homogeneous populations of pro-macromolecular switches, but could include replicate assays or assays based on heterogeneous pro-macromolecular switch populations. This arrangement is illustrated in Figure 8. The test samples could be introduced onto the microarray using fluid-handling robotics, either across the whole array, across segmented array sections or individually to each spot using, for example, ink jet printing. In this configuration an optical separation-sensitive output device such as FRET could be read using a standard microarray reader, typically based on a cooled CCD camera, reading fluorescence intensity or fluorescence lifetime measurements.. In other configurations, optical signals could be detected in optical fibres positioned into each microarray, or electrochemical signals could be detected using electrodes configured on the microarray. Ionic switching of the switchable biosensors could be achieved by introduction of ionic solutions onto the

microarray using fluid-handling robotics, either across the whole array, across segmented array sections or individually to each spot using, for example, ink jet printing. In another arrangement, ionic switching would be electrochemically generated using electrodes configured on the microarray. In a further variant, an array of electrodes could be constructed on the microarray which could be used to move fluid droplets around the array surface using electrostatics. For example, droplets of test solution and high/low salt buffer could be moved in turn onto an location upon which pro-macromolecular switches have been located, either in droplet form themselves or immobilized to the array surface. This configuration is illustrated in Figure 12.

In another arrangement, the switchable biosensors could be used in a electrophoretic separation device, including conventional gels, microfluidic biochips or polymer tape-based configurations. In this case a population of pro-macromolecular switches which has previously been mixed with solutions of test sample would be located at one end of a channel of gel matrix as decribed hereinbefore. Channels would each typically contain distinct homogeneous populations of pro-macromolecular switches, but could include replicate assays or assays based on heterogeneous pro-macromolecular switch populations. This arrangement is illustrated in Figure 10. In this configuration standard electrophoresis methods can be used whereby an electrical potential is applied to induce molecular movement along the channel through the gel matrix, due to the inherent charge of the molecules. The differing sizes and/or charges of the molecules will determine the distance moved by the macromolecular switches in each the gel channel. In this way it is possible to discriminate between pro macromolecular switches with no bound target and macromolecular switches with a bound target and this is illustrated by a standard gel assay in Figure 4. Furthermore, ionic switching of the switchable biosensors could be achieved by direct introduction of ionic solutions into the gel channels or by in-situ ion-flux generation using electrochemistry. The switching characteristics of a functional, macromolecular switch can then be detected using separation-sensitive output devices, as illustrated in Figure 4 using FRET. An optical separation-sensitive output device such as FRET could be read using standard gel scanner technology, reading fluorescence intensity or fluorescence lifetime measurements. In other configurations, optical signals could be detected in optical fibres positioned into each channel, or electrochemical signals could be detected using electrodes configured in the channel. In another arrangement, ionic switching would be electrochemically generated using electrodes configured in the gel channel.

In another arrangement, the switchable biosensors could be used as an inline detection format where a population of pro-macromolecular switches would be moved in solution or immobilized into micro-scale beads through an in-line fluidic system incorporating detectors. In this configuration an optical separation-sensitive output device such as FRET could be read using an in-line fluorescence reader, typically reading fluorescence intensity or fluorescence lifetime measurements or electrochemical signals could be detected using electrodes configured into the fluidic apparatus. Ionic switching of the switchable biosensors could be achieved by introduction of ionic solutions into the flow using valved fluid inlets or would be electrochemically generated using electrodes configured in the fluidic apparatus. This arrangement is illustrated in Figure 11.

In general, the different conformational states of the macromolecular structure of the macromolecular structure switches formed in use of the present invention will correspond to different separation distances of particular (first and second) parts of the macromolecular structure, whereby changes in conformation state may be conveniently monitored through the change in output signal from a separation sensitive output device (described in more detail hereinafter). Such devices will generally include respective output device components mounted (directly or indirectly) on relatively displaceable first and second parts of the pro-macromolecular structure switch. In the case of pro-HJ switches, such components are conveniently mounted at end portions of the HJ arms, preferably remote from the analyte binding site. In this connection we have found that the precise positioning of the separation sensitive output device components on the HJ arm end portions, can have an effect on the sensitivity and/or the resolution of the output signal reading. Example 4 shows how increasing the distance along arm length away from the branch point, at which the fluorophores are attached, increases the separation of the fluorophores in the 'open' state, reducing any background signal due to the closeness of these fluorophores when attached close to the branch point. Position of the separation-sensitive output device components is a tuneable factor in selecting optimum macromolecular switch performance. The optimum will depend on the application for which the switch is used, will vary for different macromolecular structures and may be readily determined by trial and error. For example, deliberately placing the separation-sensitive output device components close enough that there is always a background signal, even in the open configuration, may be desirable as it

forms a in-built control signal which if not present can detect malfunction of the bioswitch device.

Various forms of separation sensitive signal output device may be used in accordance with the present invention. Preferably, though, such devices are formed and arranged so as to provide discretely different output signals in said discretely different conformations of said macromolecular structure. In one preferred form of the invention there is used a FRET system comprising a first separation sensitive output device component in the form of a donor fluorophore mounted on the first part and a second separation sensitive output device component in the form of an acceptor fluorophore mounted on the second part of the macromolecule structure. In this case an optical signal is "input" into the donor to excite the donor which then transfers energy (non-radiatively) to the acceptor which then provides an output optical signal. The energy transfer from the donor to the acceptor is sensitive to the separation therebetween, thereby affecting the intensity of the output signal. In order to allow the non-radiative energy transfer to take place, the donor and acceptor fluorophores must be in relatively close proximity – generally not more than 10 nm, advantageously from 0.2 to 10 nm, preferably in the range from 2 to 8 nm. When FRET does take place, as well as being indicated by the presence of an output signal from the acceptor fluorophore which is distinguishable from any donor fluorophore fluorescent radiation by having a longer wavelength, it can also be detected by a reduction in the donor fluorophore fluorescent radiation signal level. Moreover the acceptor fluorophore output signal is quantized and binary in nature in that it provides a 0 output signal in the absence of FRET and a 1 output signal in the presence of FRET. In another preferred form of the invention, there is used only a single fluorophore type in conjunction with a quenching agent. In this case an optical signal is input into the donor which then provides an output optical signal. The intensity of the output signal in this case is sensitive to the separation between the fluorophore and the quenching agent.

It is however also possible to use non-separation sensitive fluorescent output signaling devices, which can provide quantized output signalling. Thus, for example, there may be a pro-HJ in which one of the Adenine nucleotides is replaced by a fluorophore such as 2-aminopurine (2AP) whose fluorescence is substantially quenched in the closed conformation of the corresponding HJ (but not in the open conformation), due to the influence of base stacking.

In accordance with the present invention, one embodiment of the input and output signals of the separation sensitive signal separation sensitive output device reading system, is optical signals which can readily be transmitted through an aqueous medium in which the macromolecule structure is supported in use of the device, and which can be more or less readily integrated with suitable electronic, opto-electronic, or optical signal processing elements.

The input signals to the macromolecular structure switches formed in use of the invention, for reading out analyte detection, may be produced in any convenient manner depending on the form and nature of the separation sensitive output device. In the case of a substantially electronic circuit, there may be used an electro-optical transducer, such as LEDs or lasers to produce optical radiation at a frequency suitable for use with the separation sensitive separation sensitive output device reading system (as further discussed hereinbelow). This optical radiation may be delivered from the electro-optical transducer substantially directly into the solution in which the "input connection" component of the separation sensitive output device of the macromolecular structure switch is supported, or via a suitable waveguide.

The separation sensitive output device reading system signal outputs from the switch devices in use of the invention may correspondingly be received at said another part of the output signal reading system using any suitable form of opto-electronic transducer which can collect the optical data carrier signal outputs and convert them into electrical signals for further processing.

Furthermore it is also possible to utilise non-separation sensitive systems for reading of the conformation state, including for example, systems based on changes in physical properties associated with the changes in conformation with switching of the macromolecule structure, e.g. electrochemical impedance changes generally based on changes in capacitance and/or resistance and/or inductance of a macromolecule structure population. In addition there may also be used other physical parameter monitoring systems based on structural physical changes such as a change in thickness of a macromolecule structure population layer (e.g. by dual polarization inferometry or other techniques), or as another example detection of microscale cantilever stress or resonant frequency changes.

The transformation or flipping of the macromolecule structure formed in use of the invention, between its different conformations is generally effected by applying suitable different ion binding conditions to the macromolecule structure ion binding site, depending on the general and/or particular nature of said macromolecule structure. In a preferred form of the invention the macromolecule structure comprises a Holliday-Junction structure (conveniently referred to herein as an HJ) having two pairs of arms extending out from a "branch point". In one, so-called "open" conformation the arms are fully extended or splayed out in a so-called "X-structure" which can be flipped to a "closed" or "stacked" conformation in which the two arms within each pair are substantially closer to each other than to the arms within the other pair of arms. In HJs there are generally four possible "closed" conformers (two parallel, two anti-parallel) with different combinations of arms within the pairs and unconstrained HJs typically adopt the two anti-parallel conformations (Lilley, Qly Rev Biophysics 33,2 (2000) p109-159).. It is also generally the case in naturally occurring HJs that the branch point migrates within the HJ structure. As further discussed hereinbelow, it is possible, by suitable choice of the polynucleotide sequences within the HJ branch point, to prevent substantially such migration, and this is generally preferred in order to maintain the analyte binding site in a stable and consistent form (Ref: Seeman 1982, J. Theor. Biol. 99 237-247). Similarly it is possible to favour one of the closed conformations to a greater or lesser extent, for example, by using a branch point sequence combination such as that shown in Fig 1 of the accompanying drawings. In this case the two closed conformations, iso1 and iso2, normally exist in a ratio of approximately 95: 5 (Refs: Carlstrom & Chazin, 1996, *Biochemistry* **35**, 3534 and Miick et al., **1997** *PNAS* 94, 9080). Whilst it would generally be simpler and more convenient to use HJs with just one substantially preferred closed conformation, it is nevertheless also possible to use HJs with two closed conformations. It will be appreciated that the equilibrium position adopted between each of the two closed conformations may vary based on the specific HJ branch point sequence and/or the ion used and/or the chemical composition (e.g. DNA/RNA) (Ref: Grainger et al., Biochemistry 37, 23-32 1998). In the case of using oligonucleotides macromolecular switches with more than two "closed" conformations similar comments on the equilibrium position of switching would apply.

It would also be possible to arrange the position-sensitive output device such that an output signal were given whichever closed conformation each particular molecule of the population is in. For example, with a fluorophore labeled HJ it would be possible to attach an acceptor

dye to two opposite arms of the HJ with the donor dye attached to one of the other arms so that a FRET signal would be generated whichever of the two conformations is adopted by the HJ population. It would also be possible to use multiple donors or acceptors to detect and distinguish between both conformers simultaneously.

We have also found that the ionic switching is sensitive to the efficiency and completeness of ion removal i.e. it is important to ensure that substantially all switching ion is removed to ensure a clean 'open' signal. This may be achieved for example by use of multiple spin columns (typically 99% removal) and/or use of an ion chelator such as ethylenediaminetetra(acetate) (EDTA).

Without wishing in any way to be restricted by the following, it is believed that the flipping of the HJ between open and closed conformations is affected by the ion binding conditions at an ion binding site in the region of the branch point, in at least two different ways including shielding of the negatively charged phosphate moieties of the sugar phosphate backbones within the branch point by positive ions so as to reduce repulsion therebetween, and specific binding of positive ions to the sugar phosphate backbones in the vicinity of the branch point. Hydroxyls of target RNAs in the branch point region could also participate in metal binding. Furthermore these effects can be additive. In more detail we have found that various metal cations, including monovalent ones such as sodium and potassium, and divalent ones such as magnesium and zinc, which can be used to effectively control switching, with di- or tri-valent cations being effective at significantly lower concentrations than monovalent ones.

Advantageously switching ions may be selected to optimize one or more of various practically beneficial operating features such as minimal quenching, minimal absorbance, induction of a sharp transition between open and closed conformers of a macromolecular switch, and electrochemical activity in order to enable switching using electrodes. The choice of ion to switch any particular macromolecular switch will also be dependent on the nature of the macromolecular switch used, and may readily be determined by trial and error. This may also include the use of combinations of two or more different ions. For the structures illustrated in the Examples, particularly preferred metal cations are Mg^{2+}, Zn^{2+} and Ca^{2+}. We have also found that larger cations including those which are organic to a greater or lesser degree, including the protonated spermidine cation, are also effective in controlling switching. In this case it is believed they are effective through specific binding

to the sugar phosphate backbone in the vicinity of the branch point. (On the other hand certain large cations e.g. tetramethylammonium do not appear to be effective in any way. Further suitable cations may, however, be readily determined by trial and error.) It may also be desirable in some cases to utilize ions (including buffer ions) which are electrochemically inactive and do not strongly switch the macromolecular devices at a known concentration, as such ions can be used to provide a sufficiently small screening length to act as background electrolyte for electrochemical studies and to help screen charges to optimise target hybridisation. For the HJ structures illustrated in the Examples, a particularly preferred cation is tetramethylammonium (or other tetralkylammonium ions) and a particularly preferred buffer cation is TrisH+, present in Tris/HCl buffer.

Also useful may be relatively highly charged cationic coordination complexes which are stabilised in solution by having the inner coordination shell of the metal occupied by strongly bound ligands. These systems are often electroactive, and their charge can be more or less readily varied electrochemically by changes in redox state , thereby readily facilitating electrochemical control of ionic binding conditions. This makes use of the fact that we have found there to be significant differences in ion concentration required for conformation flipping between ions of different charge. One such suitable ion system which may be mentioned is $Fe(bipy)_3^{2+}$ <- -> $Fe(bipy)_3^{3+} + e^-$. A further method for the electrochemical production of significant concentrations of redox inactive switching ions such as Mg^{2+} is the production of redox active films (such as conducting polymers) containing covalently attached or insoluble cation chelating groups such as EDTA. If the reduced redox form of the film is neutral, Mg^{2+} will be chelated in the film and the solution concentration near the film will fall. Upon electrochemical oxidation of the redox centres, an overall positive charge will be produced in the film which should lead to Mg^{2+} expulsion and a rise in the local concentration of Mg^{2+} near the film.

It will be appreciated from the above that the amount of cation(s) required will depend not only on the particular macromolecular structure used, and to some extent physical conditions (e.g. temperature), but also on the amount of macromolecular structure present. Suitable quantities in any given case may be readily determined by trial and error. Typically, though, for a macromolecular structure switch of the present invention containing 1 Micromolar of a suitable HJ, there would be required 100 micromolar of Mg^{2+} (Duckett et al EMBO Journal **9** (1990) 583-590).

It will be appreciated that various different polynucleotide sequences may be used in the HJs in accordance with the present invention. Certain types of sequence are however preferred for various reasons including one or more of: dimensional stability of the HJ structure, resistance to damage e.g. by free radicals, functioning of the data carrier signal transmission system, etc. Where a FRET system is used for separation sensitive output device output signal reading, it is preferred that that the donor and acceptor moieties are attached to the ends of the HJ arms to minimize background FRET signals, in so far as this will also reduce the level of the FRET signal generated. A convenient positioning may readily be determined for each molecular structure design by trial and error. The length of the polynucleotide sequence between the branching point and the donor and acceptor fluorophores should moreover be sufficiently long to fully avoid steric hindrance to opening and closing of the HJ, to ensure HJ stability and to ensure that data carrier signal transmission is substantially switched off in the open configuration of the HJ, without however being so long as to prevent data carrier signal transmission in the closed configuration. Where a FRET system is used, the donor and acceptor fluorophores should preferably be mounted so as to mount these at from 4 to 20 bases from the branch point, advantageously from 6 to 12 bases therefrom. It will also be appreciated that in the case of an HJ where substantial proportions of both closed conformers may be present upon switching from the open conformation, and it is desired to monitor flipping to each of the closed conformers, then an additional acceptor moiety may be provided on the other one of the HJ arms which pairs up with the Donor moiety bearing HJ arm in the second closed conformer, so that FRET monitoring may be carried out with either of the closed conformers. Moreover by using two Acceptor/Donor systems with different output wavelengths, it is possible to differentiate between switching to one or the other of the two closed conformers.

We have also found that the positioning of the output signaling system components within the pro-HJ, can also be used to control the sensitivity of analyte detection. More particularly we have found that if one component (e.g. the Acceptor) is positioned at an end of a pro-arm (i.e. single stranded analyte binding arm) rather than internally (within an end portion of a non-pro-arm), then higher sensitivity to target analyte mutations at positions which bind the Acceptor-labelled pro-arm remote from the HJ branch point, can be obtained.

In the case of an HJ anchored to a substrate via bonding of one (or more) of the HJ arms thereto, the anchored arm of the HJ is preferably of a length sufficient to provide at least one complete double-helix turn in order to provide a more stable support for the other arms. Dimensional stability may also be enhanced by using a so-called tripod mounting anchoring moiety which supports the anchored arm of the HJ in a substantially upright manner projecting out away from the substrate surface thereby reducing interference with the desired operation of the HJ structure in relation to one or more of interactions with target molecules, conformational switching etc (see for example Long B., Nikitin K., Fitzmaurice D., J. Am. Chem. Soc. 2003, 125, 5152-5160). Stability of the HJ structure may also be enhanced against separation of the polynucleotide strands of the arms, by binding them together with suitable bridging moieties.

It will be appreciated that the binding specificity of the analyte binding site will generally be dependent on the extent thereof. In the case of a pro-HJ switch, this will depend on the lengths of the polynucleotide strand sequence portions of the HJ single-stranded pro-arms. Desirably these arm portions should each have a length of at least 8 bases, preferably from 10 to 60, most preferably from 12 to 30. With covalent cross-linkage in the double-stranded arm, shorter double-stranded arm lengths less than 8 bases may be used, desirably from 4 bases, as the resultant macromolecular structure is more tightly bound in its assembled form

It will be appreciated that the various arms and pro-arms may have similar lengths or significantly different lengths, and hence that (where the pro-HJ is comprised by more than one polynucleotide strand), the strands may have various different lengths (bearing in mind also that in some cases an arm may have a non-hybridized strand sequence portion extending therefrom).

It should also be noted that HJ-like structures which have first and second, discretely different, conformations flippable in substantially similar manner to conventional HJs (consisting essentially of polynucleotide sequences), may also be constituted by molecular species in which a greater or lesser portion of the four HJ arm structures is comprised by non-polynucleotide material, and may be of any other organic and/or inorganic material which is not incompatible with the operation and application of the macromolecular structure, provided that the branch point of the HJ-like macromolecular structure has at least

the four interacting polynucleotide dimer sequence elements of an HJ. (It will be appreciated that, whilst naturally occurring HJs are comprised by polynucleotides, an HJ-like macromolecular structure may also be comprised by the corresponding polynucleoside components of polynucleotides, phosphorothioates or methyl phosphonates. Choice of these may be used to design the HJ to give the desired switching effect Ref: Liu et al., 2004 JMB 2004.) Accordingly any references to HJ structures herein are also intended to encompass such HJ-like structures unless the context specifically requires otherwise.

As discussed hereinbefore various different HJ macromolecular structures are known with differing ratios of the two possible closed conformers (see for example Hays et al Biochemistry 42 2003) 9586-97). In this connection, the nature of the dimer sequence portions at the branch point of the HJ macromolecular structure switch, is especially influential (Carlstrom & Chazin, 1996, *Biochemistry* **35**, 3534 and Miick et al., **1997** *PNAS* 94, 9080).

Another form of separation sensitive output signal device which may be used in accordance with the present invention comprises a fluorophore moiety providing input and output "connections" on said one part of the macromolecule structure and a quenching moiety provided on said second part of the macromolecule structure. In this case when the fluorophore receives energy from an exciting wavelength radiation source – corresponding to an input signal, it fluoresces sending out radiation at another wavelength – corresponding to an output signal which can be detected. The intensity of the output signal obtained is sensitive to any quenching agents which may be present in proximity to the fluorophore. Accordingly when the macromolecule structure is transformed from its first conformation to its second conformation, the quenching of the fluorescent radiation is changed thereby changing the output signal.

It will further be appreciated that quenching modulation can also be made use of in conventional FRET systems (based on energy transfer from a donor fluorophore to an acceptor fluorophore) where energy transfer to the acceptor is quenched by close interactions between the donor and another material such as, for example, DNA. In this case hybridisation of a target molecule with part of the HJ can be used to reduce such interactions thereby reducing the quenching effect and increasing the output signal from the acceptor.

It should be noted that in order to maintain the integrity of the pro-HJ structure i.e. prevent the polynucleotide sequences from separating from each other, suitable precautions should be taken. Thus, for example, the pro-HJ structure may be maintained interfaced with an aqueous medium of HJ – compatible ionic strength. Preferably the medium should be a buffer solution which contains one or more large monovalent cations at a concentration well below the concentration at which the ion switches the macromolecular structure. 20 mM Tris/HCl buffer at pH 7.5 is a particularly convenient buffer, but other buffers may also be used. Preferably the buffer pH is from 7-8. Nevertheless pro-HJ integrity may also be maintained in other ways, for example, by means of cross-linking together of the double stranded polynucleotide strands (as further discussed hereinbelow) – even under less favourable ionic strength or other conditions.

With such a Holliday Junction type of macromolecular structure, it is possible to change the ion-binding conditions so as to transform the structure between first and second conformations in various ways including physical and/or chemical perturbations such as change of ionic strength and/or composition in the aqueous medium, changing a voltage applied to the aqueous medium (including changing from no applied voltage), changing the temperature or pressure of the aqueous medium, and changing the concentration of particular organic or inorganic molecules in the aqueous medium which have specific interactions (for example multiple hydrogen bonding)– as distinct from general physical interactions such as those due to ionic strength. Moreover it is also possible to change the ion binding conditions by discretely or progressively, replacing the fluid medium in which the macromolecular structure is supported. Thus for example magnesium ions have a specific effect in switching individual HJ structures from an "open" configuration to a "closed" configuration, and are effective at significantly lower concentrations than monovalent cations such as Na^+ and K^+. In general a Mg^{2+} concentration of at least 1μM, conveniently from 10μM to 10 mM is required to switch the HJ from open to closed configurations. Various other divalent and multi-valent cations may also be used in this way. Species such as Zn^{2+} which is redox active through the redox reaction $Zn <=> Zn^{2+} + 2e-$, may be used advantageously as Zn^{2+} is soluble and preferentially induces HJ switching over insoluble Zn at a defined concentration, thereby enabling conformational switching electrochemically by means of changing said species from one redox state to another. Other switching cations which may be mentioned include Al(III), which can be electrochemically generated from insoluble Al, and Hexamminecobalt (III) $[Co(NH_3)_6^{3+}]$, which allows electrochemical switching as it has a

lower switching concentration than the soluble hexamminecobalt (II) [$Co(NH_3)_6^{2+}$] produced by electrochemical reduction.

It is also possible to transform the biological macromolecular structure between said first and second conformations, by means of more or less specific interactions with positively charged biomolecules. Thus for example an HJ structure can also be transformed from its open to its closed conformation in the presence of protonated polyamine species such as the protonated spermidine cation.

In the case of other biological macromolecule structures involving one or more of macromolecule structures such as DNA, RNA, peptides, polypeptides, proteins, polysaccharides, and complexes formed by interactions between any of these, it may be possible to change ion binding conditions so as to switch conformations by various different means such as for example ionic strength and composition, solvent composition, light excitation, pH, molecular binding, redox switching and electric fields. Various specific examples of macromolecule structures which would be suitable are already known, see for example Rosengarth & Luecke (J Mol Biol 2003 Mar 7;326(5):1317-25)relating to the conformationally switchable polypeptide Annexin A1, and Backstrom et al (J Mol Biol 2002 Sep 13; 322(2):259-72) relating to the conformationally switchable polypeptide RUNX1 Runt. Various other suitable macromolecular structures with specific properties may be more or less readily obtained by means of standard iterative screening and amplification such as SELEX (systematic evolution of ligands by exponential enrichment) which is used with polynucleotide (including DNA and RNA) sequences, and Phage Display which is used for polypeptide sequences. (see for example Methods Enzymol. 364 (2003) 118-142)

Suitable Polypeptide macromolecular structure switches according to the present invention also include inter alia metallo-enzymes that have discretely different conformations between which it is flippable upon binding to an ion.

Particularly preferred macromolecular structures according to the present invention are those comprising at least one polynucleotide, and most preferably those comprising a Holliday Junction. Particularly suitable other (Non-HJ based) pro-switch macromolecular structures which could be used in accordance with the present invention include 3 way nucleic acid junctions (Ref: Welch et al., NARS 1993, 21, 4548-4555, 1993), and zinc fingers (further

described below). Another type of macromolecular structure which may be used in accordance with the present invention is an ion-induced polysaccharide conformational switch such as E.coli K1 capsular polysaccharide (Koenig and McLean, Biometals Vol 12, Number 1/March, 1999) or other synthetic polysaccharides e.g. Henry et al, Carbohydrate Research 315 (1999) 48-62.

With regard to the provision of biomolecule analyte binding sites, in the case of HJ macromolecular structures, these can conveniently be in the form of so-called "aptamer" polynucleotide sequences which function as receptors for specific target biomolecule analytes from a wide range of biomolecule types including not only nucleotide sequences, but also polypeptide sequences, and which aptamers are obtainable by use of the well known SELEX in vitro selection technique in which very large numbers of random polynucleotide sequences are iteratively screened and amplified for binding to a particular target biomolecule analyte. In the case of a polypeptide macromolecular structure switch, suitable polypeptide analogue biomolecule binding aptamers are obtainable by use of well known in vitro selection techniques such as Phage Display.

One well known form of macromolecule structure which may be mentioned here is the so-called "zinc finger" which comprises a polypeptide which has a conformationally transformable portion which switches from a generally straight extended configuration into a loop which can bind DNA (see for example Wolfe et al Biochemistry **42** (2003) 13401-9). In this case the first and second, portions of the separation sensitive data carrier signal transmission portion (e.g. donor and acceptor fluorophores of a FRET system) are mounted on first and second parts of the zinc finger structure at opposite end portions of said conformationally transformable portion so that they are moved towards and away from each other as the zinc finger switches conformation.

A zinc finger involves the coordination of a metal ion with 4 amino acid residues at a metal coordination site - the amino acid residues for metal coordination being cysteine (C) and histidine (H). For the purposes of the pro Zn Finger - the zinc coordination site is split between two separate polypeptide arms. Each arm is comprised by two functional modules. In a given arm, one module is comprised of either one half of the metal coordination site (ie the C2 or H2 moiety) and the other module of the protein interaction domain. Labels for donor and acceptor are positioned on the polypeptide arms such that FRET will only be

detected when a functional Zn finger coordination occurs and which prefers/requires intramolecular association. This will occur only if correct and specific association between binding modules occurs. Note that a restriction of such interactions is that they must be compatible with the formation of the zinc coordination centre (ie cannot be too bulky as to cause steric hindrance).

An example of a protein interaction domain that would be feasible in the current invention is a leucine zipper (typically 6 - 24 amino acids long). There are over 300 members of this family and the association of a specific member is determined by its partner interaction. A pro-switch could be formed using a "bait and prey" configuration for detecting protein-protein interaction (Fields, S. and Song, O.. Nature. **340** (1989) 245-6). Therefore one arm could include a 'bait' moiety - this a fixed probe sequence, and the other arm the 'prey' moiety(the target). A 'bait' epitope would be made synthetically and assembled together with an attached polypeptide tail of 10-20 amino acids with cysteine (C) residues at either end, which is labeled with a donor fluorophore (D). A sample containing the 'prey' epitope or target, which could be a naturally occurring protein or a synthetic moiety depending on application, could be labeled with a polypeptide tail of 2-4 amino acids with histidine (H) residues at either end, and with an acceptor fluorophore (A) attached. (Alternatively this acceptor labelled moiety could be attached post target binding, forming a label-free sandwich-assay system). This system is illustrated in Figure 3.

In this assay the bait and prey epitopes would selectively bind together upon specific leucine zipper partner association, the combined arms together forming a metal ion induced switch in conformation mediated by the Zn finger moieties. In the presence of Zn^{2+} ions this structure would form a zinc finger due to the specific chelation of the polypeptide tails, and a FRET signal could be generated. In low salt conditions, the macromolecular structure would adopt a more open conformation and there would be a significantly lower FRET signal.

It will be appreciated that in the case of oligomeric macromolecular structures according to the present invention, said first (open) and second (closed) conformations correspond to more or less narrowly defined stable states, whereby said transformation therebetween operates in an essentially quantized, binary (or ternary etc in some cases) or digital manner thereby yielding binary or digital output signals (0 or 1 etc) from said signal transmission portion. Whilst it is a particular feature of the invention that the conformational flipping of

the macromolecular structures can provide a substantially quantized output (with discretely different output signal values), it is also possible to use them to obtain analogue-form output signals. Thus, for example, analogue-type operation can be obtained in situations where the number of target molecules is significantly smaller than the number of macromolecule structures so that a positive output signal is obtained only from some of the macromolecule structures whereby the intensity of the output signal obtained depends on the concentration of the target molecule.

It will be appreciated that the macromolecular structure switches formed by binding of the pro-switches of the present invention with target analytes, may, in principle, signal out the detection of the target analyte, in a variety of different ways.

It should be noted that, in general, in at least some cases, depending on *inter alia* the nature of the macromolecule structure, transformation or flipping between different conformations may be obtainable by two or more different control signal inputs affecting ion binding conditions, for example, voltage and ionic strength, so that various combinations of different control signal inputs may be used which may have additive and/or subtractive effects (which may moreover be multi-dimensional), on the conformation transformations. Thus the effect of any given control signal input will also depend to a greater or lesser extent on any other (secondary) control signal inputs which may be present. This can also allow, for example, the sensitivity of the conformation device operation, to any desired control signal input, to be adjusted or tuned, by means of suitable choice of one or more other control signal inputs (and strength of biomolecular binding). In general the macromolecular structure switch is preferably configured so that the ionic binding conditions immediately before macromolecular structure switching, are close to the switching threshold for the ionic binding condition variable(s) being used to effect switching, in order to minimize the change in ionic binding conditions required for switching. In another preferred form of the invention discussed elsewhere herein, the macromolecular structure may be pre-biased towards a switched conformation, against the restraint of a conformation "locking" device.

It will be appreciated that a variety of different kinds of separation sensitive output device may be used in the macromolecular structure switches of the invention. One particularly convenient form of system is fluorescence resonance energy transfer (FRET) in which the input and output connections comprise donor and acceptor fluorophores. Suitable donor

fluorophores which may be mentioned include Cyanine 3 (Cy3) , FAM (carboxyfluoescein) and Fluorescein which are readily available commercially. Suitable acceptor fluorophores include Cyanine 5 (Cy5), TAMRA (carboxytetramethylrhodamine) and Rhodamine (Tetramethylrhodamine) which are readily available commercially. Cy3 has an excitation wavelength of 552 nm and Cy5 provides a maximum output radiation at 673 nm. In general, with such signal transmission, a suitable light source is used to supply radiation at a first wavelength to the donor fluorophore which transfers energy non-radiatively. If the acceptor fluorophore is within a predetermined range of the donor fluorophore (typically from 10 to 100 Angstrom), then the acceptor fluorophore will be able to convert energy received from the donor, into an output signal at a second wavelength which can be detected using suitable imaging spectroscopy apparatus.

Thus detection of an output signal at said second wavelength will indicate the presence of acceptor and donor fluorophores in the complex, in a conformation of said macromolecule structure in which the fluorophores are sufficiently close to each other for energy transfer therebetween. (Additional more detailed information on the separation between the donor and acceptor fluorophores, and/or for example the decay rate of fluorophores due to quenching thereof by other moieties (thereby enabling the use of single-fluorophore systems), can be obtained, if desired, by the use of more sophisticated output signal imaging analysis techniques such as frequency domain fluorescence lifetime imaging microscopy (FLIM)(Lacowicz, Principles of Fluorescence Spectroscopy, Princeton University Press) and Total Internal Reflection Fluorimetry (TIRF).

Various other forms of separation sensitive output device designed to give quantized output may also be used in accordance with the present invention including electrochemical, where for example the environment around the redox-active active site of an enzyme tethered to one of the arms of the biomolecule can be altered by the change in conformation of the biomolecule, designed to induce a relative movement of an enzyme inhibitory substance tethered to the other arm. This would affect the enzyme reactivity, and hence the catalytic current due to the enzyme reaction, which could produce an electrochemical signal output. Such a catalytic system would also provide signal output amplification. Similarly, separation sensitive photoinduced charge transfer between, for example, a fluorophore and a quencher could be detected electrochemically.

In general there can be obtained a substantial change in the HJ conformation between the first and second conformations, resulting in a relatively large change in the separation of the signal transmission device component fluorophores (for example a change from a separation of the order of 1 nm to a separation of the order of 10 nm) so that two clearly distinct signal outputs can be reliably obtained, thereby providing a binary signal transmission capability, suitable for use in computational devices. It will be appreciated in this connection, that the sensitivity of any such separation sensitive readout system can be maximised if required, by careful choice of the positioning of the interacting components thereof on the relatively displaceable parts of the macromolecular structure. The precise optimal positioning will vary for different structures (for example different arm lengths may cause a rotational difference in the position of the fluorophores due to the double helix structure) and this can readily determined by trial and error. The effect of varying dye position is further discussed hereinbelow in the Examples.

Preferably fluorescent dyes should not be positioned next to a guanine residue to avoid quenching (Nazarenko et al., 2002 Nucleic Acids Res, 30, 2089).

Whilst in general pro-HJs are not capable of flipping between discretely different conformations, in some cases they can nevertheless produce low level signals with separation sensitive detection systems (such as, for example, FRET) upon changing of ion binding conditions Typically though such signals are less than 30 % of the signal obtained with the corresponding HJ, and thus can readily be distinguished. Furthermore, it is possible to minimize this by positioning the fluorophores at increasing distances from the branch point, along the respective pro-HJ arms.

As noted above, various different kinds of separation sensitive signal transmission system may be used in the macromolecular structure switches of the invention as already discussed hereinbefore. The signal connections used will naturally depend on the nature of the system. Thus, for example, where a FRET system is used, the connections would conveniently be in the form of optical waveguides optically coupled to the switch material.

The liquid medium mediated input signal may take various different forms. Thus for example in the case of a data carrier signal transmission system based on an enzyme or other biocatalyst in the HJ, then the input signal could be a mediator (e.g. NAD+/NADH,

[Fe(CN)6]3-/4- or Fc/Fc+ where Fc is a soluble substituted ferrocene such as ferrocene carboxylic acid. In this case signalling in/out may be effected via two electrodes separated spatially either side of the HJs, one electrochemically generating mediator and the other detecting or collecting mediator which had undergone redox reaction (both electrodes could be held at the same potential). One example of such a system uses Fc and GOD (glucose oxidase) as the enzyme. Given a large amount of glucose substrate, the rate of activity of the enzyme may be measured by the amount of Fc produced from electrogenerated Fc+, which is given by the current due to Fc ----> Fc+ at the collector electrode.

Although the devices of the present invention will normally be employed with said pro-switches supported in an aqueous medium, it may be more convenient for storage and/or shipping purposes to present the devices in a lyophilised form, and accordingly the present invention also encompasses such forms thereof.

It is envisaged that the invention may be used in a number of applications where the specific detection of target molecules, in particular nucleic acids, is a key feature. It is a particular advantage of the invention that the switchable macromolecular structure must both form AND it must display ion-controlled switching, in order to give a positive detection signal of the target molecule, as demonstrated in the following Examples. Detecting transition between different states depending on these two inputs allows a greater degree of interpretation about the target/pro-switch binding to be gained, and a greater degree of certainty about the interpreted result. This gives the specific benefit of a more definitive signal of target analyte detection, and less chance of false positive signals due to non-specific binding.

This feature gives critical advantages in specificity over simple complementary base-pair hybridization. For example in one particular application of the invention comprising transcript detection, the available interpretations might comprise:

Before target addition	Post target addition	When ion signal applied	When ion signal removed	Interpretation
Open	Open	Open	Open	Target not present
Closed	Any	Any	Any	Device failed

Open	Closed	Any	Any	Non-specific binding
Open	Open	Closed	Closed	Non-specific binding
Open	Open	Closed	Open	Target presence confirmed

In this case, a set of non-specific binding events which could have given rise to false positives using other detection technologies, are clearly distinguished and can be discarded. Preferentially, switchable biosensors will switch reversibly, so that the full characteristics of the macromolecular switch formed can be repeatedly tested, adding further robustness to the measurement Switchable biosensors are therefore more specific than the equivalent single-stranded oligonucleotide hybridization probes.

This feature leads to a specific advantage of the invention, namely the ability to detect target molecules with reduced false positive signals due to non-specific binding. For example, traditional single-stranded nucleic acid probe design involves a compromise between two factors, Short oligo probes (typically 20-30mer) are specific but less sensitive as they have fewer hydrogen bonds per molecule at a certain stringency, and long oligo probes (typically 50-70mer) which have increased sensitivity but suffer from non-specific binding due to target loops and partial hybridization. The resulting trade-off results in either using large numbers of short oligo probes to detect a single target, which increases sensitivity while retaining specificity, or by careful design of a long oligo probe to reduce non-specific binding. As a result it is also necessary in some applications to use multiple long probes, which are used in combination based on a scoring or voting system to screen out non-specific binding events. As switchable biosensors are based on both target binding AND formation of a functioning macromolecular switch, they can screen out potential false positive signals, as described above. Therefore, for example, a long nucleic acid detection sequence could be used to enhance sensitivity of detection without the same false positive signals due to non-specific binding that would be experienced with a single-stranded oligo of equivalent length, and the enhanced sensitivity means that fewer probe molecules are required to detect a given number of target molecules, and hence less probe material can be used. Alternatively, in another application, probes of shorter length than the equivalent

single-stranded oligonucleotide hybridization probes could be used, simplifying oligo design and manufacture. In cases where multiple probes are used to screen out non-specific binding, the number of probes used can be reduced.

Another particular advantage of the invention is the ability to detect target molecules without prior labeling of the target sample. In one embodiment, a pro-HJ is provided with a separation sensitive output device comprising a FRET dye pair (Figure 1). Specific detection of an unlabelled target molecule results in the formation of the HJ switch, which can then be detected by operating the switch and sensing the FRET signal. By labelling the pro-HJ rather than the target, this approach offers benefits over other label-free techniques such as impedance, in that it retains the sensitivity benefits of a labelled detection system. This is further illustrated in Example 1.

We have also found that it is possible to form hybrid macromolecular structure switches, for example using a pro-HJ synthesized from DNA to specifically detect target RNA sequences, with high specificity and high sensitivity, without the need for analyte labeling. In this way it is possible to form arrays of switchable biosensors which will detect both DNA and RNA sequences by means of the use of a pro-macromolecular structure switch populations which binds together with a different components of the target analyte mixture, so as to form a functional macromolecular structure switch such as those described in our earlier WO2004/099767.

Branch point sequence is critical for the switching characteristics of the HJ switch, and in particular determines the ion binding affinity, and the thermodynamic equilibrium of the HJ conformers (Carlstrom & Chazin, 1996, *Biochemistry* **35**, 3534 and Miick et al., **1997** *PNAS* 94, 9080). This provides the ability to recognize nucleotide mismatches at a specific position in the specifically bound target sequence which results in the combined benefits of specific sequence location together with specific base mutation detection. Switching characteristics are also influenced by the chemical composition at the branch point, and it is therefore possible to distinguish between DNA and RNA target sequences based on the different switching characteristics of the macromolecular switches formed.

It is also recognized that using a electronic control system to control reversible switching using an electrode mounted in contact with the solution of macromolecular switches would

enable the reading of biodetection events over time. Given the kinetics of macromolecular switching this monitoring could also take place in real time.

Given these features, It is envisaged that switchable biosensors will have specific benefits in biological research, drug development and screening, diagnostic testing and as components of in-vivo medical devices.

The invention has particular application in the field of transcription profiling, where multiple analytes are detected in a single sample. It has advantages over existing techniques in the field, such as microarrays, in that it is free of sample labeling, it is less prone to false positives due to non-specific binding, can be used to monitor biodetection over time and can detect analytes of different types e.g. DNA, RNA, proteins.

The invention also has particular applications in the detection of single nucleotide polymorphisms (SNPs). The switching characteristics of an HJ formed from a pro-HJ is particularly sensitive to the specific sequences at the HJ branch point, and so a single base pair mismatch can be detected, again in label free conditions. As mentioned above the invention is also particularly advantageous in detecting a particular SNP in a region of DNA with other closely clustered SNPs. There is also the potential of using single or small populations of pro-HJs which may allow detection of SNPs without the amplification required for most current SNP detection techniques. Current PCR techniques for DNA amplification prior to SNP detection are also dependent on accurate recognition of three or four primer sequences – this invention offers advantages of only relying on a single target recognition site, resulting in a more robust assay with fewer points of potential failure. An illustration of this application is provided in Example 8 and Figure 16.

The invention also shows particular benefits for the detection of RNA splicing. In this application, it would be preferable to design an analyte binding site which corresponds to a target sequence that spans the junction between two exons – as illustrated in Figure 5, preferably with the branch point aligned with the boundary between the two exons. This would offer benefits over , for example, current microarray-based approaches use multiple oligonucleotide probes for gene splicing which use separate probes to detect the presence of the exon (positive control), the intron (negative control) and the junction points (test). Separate probes are used to avoid problems of non-specific binding that would occur with a

single long (50-70mer) probe. Switchable bioswitch detection enables the use of a single probe to detect this splice junction, as in this case a successful positive result will only arise on the formation of a HJ with four double stranded arms. The measurement of the switching characteristics described above would result in fewer false positives due to non-specific binding . Similarly such a construction can be used to detect DNA insertions or deletion break points in which the target binding site spans the junction between the translocated DNA molecules - as illustrated in Figure 6, with the branch point is aligned with the break point.

It will be appreciated that the binding specificity of the analyte binding site – as indicated by hybridisation stringency in relation to HJ or HJ-like macromolecular structures, will generally be dependent on a number of variables. In the case of a pro-HJ switch, this will include inter alia the lengths of the polynucleotide strand sequence portions of the HJ pro-arms, and the base composition (ratio of G/C to A/T bases) within them, and their chemical composition e.g. DNA/RNA, as well as the presence of organic solvents and the extent of base mismatching, the combination of the various parameters being more important than the absolute measure of any one.

In this connection it may be noted that the various different relative levels of stringency are generally understood in the art to have the following meanings. "Low" or "reduced stringency" refers to hybridisation and wash conditions of 6xSSC (standard sodium citrate) at 65°C. "Normal stringency" refers to hybridisation and wash conditions of 2xSSC at 65°C while "high stringency" refers to hybridisation and wash conditions of 0.1xSSC at 65°C. Alternatively, hybridisation may occur in the presence of 50% formamide at 42°C. Generally, stringent conditions are selected to be approximately 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridises to a perfectly matched probe. Typically for hybridisation, stringent conditions will be those in which the salt concentration is at least about 0.02 molar at pH7 and the temperature is at least about 60°C.

Typically, pro-HJ target binding will require high stringency target binding, affording a high level of specificity. However, once the HJ is formed, further specificity can also be provided

in lower stringency conditions due to the detection based on ionic switching characteristics. As described above, the invention provides significant advantages over existing single-stranded oligo probes in which a compromise is made between sensitivity and specificity which results in lower fidelity signals. Switchable biosensors would allow greater sensitivity of detection over existing technology platforms.

In practice we have found that the sensitivity of target detection and discrimination between matched and mismatched targets for various target : (pro-HJ) probe combinations can be substantially enhanced by means of optimization of key variables (such as: target and probe concentrations, ratio of target:probe concentrations, and switching ion concentration, and temperature, and time) involved in binding of the target and switching of the HJ devices formed by binding of the target to the probe, in use of the invention. As shown in the following Examples, it is possible to increase both the stringency of the target binding conditions and the stringency of the switching conditions, to provide a dramatic increase in sensitivity.

Hybridisation kinetics may also be a factor. For example existing methods of nucleic acid detection typically require hybridization at elevated temperatures over several tens of minutes. Pro-HJ devices according to the present invention can be expected to bind target analytes and switch at room temperature within minutes, an improvement over current methods which require annealing at elevated temperature.

In relation to the present invention, the most practically significant variable will generally be the branch point sequence. Branch point sequence is critical for the switching characteristics of the HJ switch, and in particular determines the ion binding affinity, and the thermodynamic equilibrium of the HJ conformers (Miick et al., **1997** *PNAS* 94, 9080). In this particular application of pro-HJ structures for the detection of a single nucleotide difference, the invention is less dependent on arm length for sequence specifity, because detection is more reliant on the switching characteristics of the particular branch point formed than complementary base pair binding of the target/single stranded pro-HJ arms. However, as above, for selective target binding these HJ arms should have a length of at least 8 base pairs, preferably from 10 to 60, most preferably from 12 to 30.

It will be appreciated that the various arms and pro-arms may have similar lengths or significantly different lengths, and hence that (where the pro-HJ is comprised by more than one polynucleotide strand), the strands may have various different lengths (bearing in mind also that in some cases an arm may have a non-hybridized strand sequence portion extending therefrom).

It will be appreciated that other factors will also generally need to be taken into account in the design of a pro-HJ for a particular analyte of interest, and in particular in the design of the sequences of the HJ pro-arms, such as for example in order to avoid self-hybridization as a result of different portions of the two pro-arm sequences exhibiting self-complementarity. In practice though such restrictions can be more or less readily overcome insofar as a typical mRNA transcript target analyte may be some 2000 base pairs in length, and so there are many different stretches of complementary 25mer (say) oligonucleotide that could be selected to specifically detect this gene.

It should also be noted that HJ-like structures which have first and second, discretely different, conformations flippable in substantially similar manner to conventional HJs (consisting essentially of polynucleotide sequences), may also be constituted by molecular species in which a greater or lesser portion of the four HJ arm structures is comprised by non-polynucleotide material, and may be of any other organic and/or inorganic material which is not incompatible with the operation and application of the macromolecular structure, provided that the branch point of the HJ-like macromolecular structure has at least the four interacting polynucleotide dimer sequence elements of an HJ. (It will be appreciated that, whilst naturally occurring HJs are comprised by polynucleotides, an HJ-like macromolecular structure may also be comprised by the corresponding polynucleoside components of polynucleotides.) Accordingly any references to HJ structures herein are also intended to encompass such HJ-like structures unless the context specifically requires otherwise.

As discussed hereinbefore various different HJ macromolecular structures are known with differing ratios of the two possible closed conformers (see for example Hays et al Biochemistry 42 2003) 9586-97). In this connection, the nature of the dimer sequence portions at the branch point of the HJ macromolecular structure switch, is especially influential.

As noted above, the present invention is applicable in relation to various different kinds of macromolecular structure switch. One well known form of macromolecular structure switch which may be mentioned here is the so-called "zinc finger" which comprises a polypeptide which has a conformationally transformable portion which switches from a generally straight extended configuration into a loop which can bind DNA (see for example Wolfe et al Biochemistry 42 (2003) 13401-9). In this case the first and second, portions of the separation sensitive data carrier signal transmission portion (e.g. donor and acceptor fluorophores of a FRET system) are mounted on first and second parts of the zinc finger structure at opposite end portions of said conformationally transformable portion so that they are moved towards and away from each other as the zinc finger switches conformation.

It will be appreciated that a polynucleotide designed for traditional hybridising with a particular target DNA, can also hybridise with a variant of that DNA in which one or two bases are no longer matched properly resulting in a lesser degree of hybridisation and hence weaker binding. In such a case the variant of the target DNA might still form a functional macromolecular structure switch, and thereby provide a positive output signal. Where it is desired to detect the presence of a maximal match with a target DNA using an HJ type macromolecular structure – as in the case where single nucleotide polymorphisms (SNPs) and the like are present – then it is desirable that the target analyte-binding portions of the pro-switch should be formed and arranged so that, when the target analyte is bound to the pro-switch, the SNP is located at the junction portion of the HJ type macromolecular structure, as the switching characteristics of the HJ switch are especially sensitive to the specific base sequence in this region.

In a preferred aspect the present invention provides a pro-HJ switch
suitable for use in detecting a target analyte comprising an oligonucleotide substantially characteristic of said analyte, wherein said pro-HJ switch has an oligonucleotide-binding site formed and arranged for binding said oligonucleotide with high specificity so as to form together therewith,
an HJ switch
having an ion binding site, and
flippable between a plurality of discretely
different conformations

corresponding to different ion binding conditions
at said ion binding site,
at least in the absence of any other binding to said HJ,
wherein said discretely different conformations are capable of
providing, in use, with an output signal reading system, characteristic output signals and wherein said pro-macromolecular structure switch is substantially incapable of being flippable between said plurality of discretely different conformations and providing, in use, with said output signal reading system, said characteristic output signals.

Thus in a further aspect the present invention provides:
a pro-HJ macromolecular structure switch suitable for use in detecting an analyte containing an SNP,
wherein said pro-switch has an analyte binding site formed and arranged for binding said analyte with high specificity so as to form together therewith,
a macromolecular structure
having at least one ion binding site, and
flippable between a plurality of discretely
different conformations
corresponding to different ion binding conditions
at said at least one ion binding site,
at least in the absence of any other binding to said macromolecular structure,
wherein said discretely different conformations are capable of
providing, in use, with an output signal reading system, characteristic output signals and wherein said pro-macromolecular structure switch is substantially incapable of being flippable between said plurality of discretely different conformations and providing, in use, with said output signal reading system, said characteristic output signals; and wherein said analyte binding site is formed and arranged for binding said analyte so that said SNP binds to the pro-HJ switch at the junction region of said HJ switch.
Preferably said pro-switch is provided with at least one separation sensitive output device formed and arranged for providing different output signals in said discretely different conformations,

In another preferred aspect the present invention provides: a pro-HJ macromolecular structure switch suitable for use in detecting gene splicing, wherein said pro-switch has an

analyte binding site which corresponds to a target sequence that spans the junction between two exons, preferably with the branch point aligned with the boundary between the two exons.

In another preferred aspect the present invention provides a method for detecting a target analyte comprising an oligonucleotide substantially characteristic of said analyte, in a sample, which method includes the steps of:

providing a pro-HJ switch,

wherein said pro-HJ switch has an oligonucleotide binding site formed and arranged for binding said oligonucleotide with high specificity so as to form together therewith,

an HJ switch

having an ion binding site, and

flippable between a plurality of discretely

different conformations

corresponding to different ion binding conditions

at said ion binding site,

at least in the absence of any other binding to said macromolecular structure,

wherein said discretely different conformations are capable of

providing, in use, with an output signal reading system, different characteristic output signals, and wherein said pro-HJ switch is substantially incapable of being flippable between said plurality of discretely different conformations and providing, in use, with said output signal reading system, said different characteristic output signals;

contacting said pro-HJ switch with a said sample under binding conditions, so as to form a said HJ switch with any said target analyte present;

providing a said output signal reading system;

applying an external input signal to said at least one input device so as to change the ion binding conditions at said ion binding site so that said macromolecular structure switch is flipped from a first said conformation to a second said conformation; and

reading out any change in said output signal.

In one preferred form of the above method of the invention, for the detection of gene splicing, there is used a pro-HJ which has an analyte binding site which corresponds to a target sequence that spans the junction between two exons, preferably with the branch point of the corresponding HJ, aligned with the boundary between the two exons.

In another preferred form of the above method of the invention, for the detection of an SNP, there is used a pro-HJ which has an analyte binding site which corresponds to a target sequence that includes the SNP, wherein the branch point of the corresponding HJ is aligned with the SNP.

In yet another preferred form of the above method of the invention, for use in transcript profiling, there is used a multiplicity of pro-HJs with analyte binding sites which correspond to a multiplicity of different target sequences.

In yet another aspect the present invention provides: a pro-HJ oligonucleotide suitable for use as a pro-HJ structure for binding with a target oligonucleotide, said pro-HJ oligonucleotide comprising:
first and second, target binding, portions, each having a base sequence such that together they are complementary to the base sequence of said target oligonucleotide, whilst being substantially non-self-complementary;
third and fourth portions separated by a fifth, linkage, portion, said third and fourth portions being complementary to each other when folded back across each other about said fifth linkage portion; and
sixth and seventh portions separated by a eighth, linkage, portion, said sixth and seventh portions being complementary to each other when folded back across each other about said eighth linkage portion,
said third to eighth portions extending serially between said first and second portions.
Conveniently said first and second portions are respective terminal portions of the pro-HJ oligonucleotide.

Further preferred features and advantages of the invention will appear from the following detailed examples and drawings provided by way of illustration.

Brief Description of Drawings

Figure 1 – Bimolecular Pro-HJ switch

Figure 2 – Trimolecular Pro-HJ Switch With Covalent Cross Linkage

Figure 3 – Peptide Zinc Finger Macromolecular switch

Figure 4 – Gel-FRET assay showing independent processes of ion induced switching and target binding

Figure 5 – Detection of RNA Splicing

Figure 6 – Detection of DNA Breakpoint

Figure 7 – Multi-well plate Apparatus

Figure 8 – Microarray Apparatus

Figure 9 – Microwell/Microbead Array Apparatus

Figure 10 – Electrophoretic separation apparatus

Figure 11 – Inline detection apparatus

Figure 12 – Droplet Electrode Array Apparatus

Figure 13 – Switching characteristics shown as FRET ratio as a function of $MgCl_2$ concentration in the presence (solid) and absence (hollow) of complementary DNA strand.

Figure 14 Alternative pro-HJ designs with internal dye labelling

Figure 15 Alternative dye positions

Figure 16 – Single nucleotide polymorphism (SNP) detection

Figure 17 – Shows schematically Open and Closed positions of HJ

Figure 18 – Detection of gene sequences using HJ probes for Ccl5 and Cxcl9. Pro-HJs designed with Ccl5 or Cxcl9 as target have been analysed by gelFRET in the presence or absence of their respective target DNA sequences (+/-). Analysis was performed in the presence of 5mM

Figure 19–Schematic representation of Mg^{2+} induced switching of HJ A-2131935C and A-2131935A

Figure 20–Detection of specific alleles using HJ probes for SNP A-2131935. Pro-HJ's designed with sequences of A-2131935 C or A alleles have been analysed by gelFRET in the absence or presence of perfect match and mismatch alleles (- (no target), G (G target oligo) or T (T target oligo)). Analysis was performed in the presence of 5mM $MgCl_2$. FRET ratios are shown below the band.

Figure 21– Use of 2AP output signaling system

Figure 22– 2AP fluorescence with and without target in the presence and absence of Mg

Figure 23– Effect of mismatch separation from HJ branchpoint on output signal

Figure 24– Multi=array slide for use in comparing ion binding conditions

Figure 25– The change in FRET ratio with ion buffer exchange

Figure 26– The corresponding FRET ratio observed as a function of oxidation charge (+ charge values, corresponding to increasing Zn^{2+} concentration) and reduction charge (- charge values, corresponding to decreasing Zn^{2+} concentration)

Figure 27– Effects of position of output signalling system components in pro-HJ on output signal

Figure 28– Pro-HJ:Target complex used with time-resolved fluorescence measurement output signal system

Figure 29– Use of time-resolved FRET decay output signal system

Figure 30– Use of multiple output signal systems

Figure 31– Effect of analyte binding temperature on output signal

Figure 32 – Effect of buffer conditions and probe concentrations on target discrimination

Figure 33 – Effect of high stringency binding conditions on target discrimination

EXAMPLES

1. (A) Preparation of pro-HJ Structures and (B) Use of pro-HJ Structures for Label-free detection of synthetic DNA oligonucleotide

A. We have constructed a population of Pro Holliday Junction (Pro HJ) macromolecular structures using well-known techniques ("Nucleic Acids in Chemistry and Biology", 2nd Edition, Blackburn G.M. and Gait M.J.,). Pro HJ structures comprise a single polynucleotide (as shown in Figure 1) or 3 separate polynucleotides (as shown in Figure 2) with suitably chosen sequences such that respective inverted repeat or end portions bind together, and in the case of separate polynucleotides may be covalently or non covalently crosslinked, so as to produce a Pro HJ structure with two double stranded arms and two single stranded arms radiating out from a branching point. In more detail the polynucleotide sequences are based on similar known sequences (Kallenbach N.R., Ma R.I., Seeman N.C. (1983) Nature 305 829-830), and are selected so as to provide an immobile junction which substantially prevents migration of the branching point. The polynucleotides have additional fluorescent dye moieties, Carboxyfluorescein (FAM) and Tetramethylrhodamine (TAMRA), attached either internally to the polynucleotide sequence or externally at the 5' end (Eurogentec, Belgium), to act as fluorescence resonance energy transfer (FRET) donor and acceptor moieties. The sequences present in the single stranded arms are designed to bind to a specific target polynucleotide sequence and have minimal self complementarity. When this Pro HJ molecule is hybridised to the complementary target sequence an immobile four-way junction is formed which is capable of undergoing a conformational transition by binding cations

such as Mg^{2+}. A process of heat induced denaturation followed by a slowly ramped cooling to allow annealing of complementary sequences present in the Pro HJ double stranded arms is used to assemble the Pro HJ structures as described below. By providing in this way a FRET donor and acceptor moiety on each of the proHJ structure arms, it will be appreciated that when the HJ structure is switched (as further described hereinbelow) from its "open" conformation shown in Fig. 17 in which the arms are all spaced apart, to either of the possible "closed" conformations shown in Fig. 17, the FRET donor will be brought into close proximity with a FRET acceptor. The aim of this design is to create a molecule with minimal intrinsic ion-induced conformational switching which on addition of a target molecule creates a functional macromolecular switch. The ion-induced switch characteristic of this DNA construct can be observed using Fluoresence Resonance Energy Transfer FRET between a donor (FAM) and an acceptor (TAMRA) only when the probe and target molecule are annealed to form a complete HJ.

B. The buffer for assembly (i.e. binding of pro-HJ to target so as to form HJ) reactions was 20 mM Tris/HCl (pH7.5), with 5 mM $MgCl_2$, 50mM NaCl. For assembly, pro HJ oligonucleotides were mixed with or without target oligonucleotides and the assembly buffer detailed above and the mixture was heated to 80°C for 30 minutes in a water bath and then allowed to cool to room temperature, while still jacketed by the water bath inside a polystyrene box to ensure a slow temperature ramp. The junctions were initially suspended in 20mM Tris/HCl (pH7.5), 5mM $MgCl_2$, 50mM NaCl and the final step in the sample preparation was to remove the Mg^{2+} and Na^{+} ions by sequential application to two gel filtration columns to buffer exchange the sample into 20mM Tris/HCl (pH7.5) in order to ensure HJ's are in an open conformation.

When this molecule is hybridised to the complementary (target analyte) strand (GCATAGTGGATTGCA) necessary to form an immobile four-way junction it forms a functional macromolecular switch which undergoes a conformational transition by binding magnesium ions. A 1μM solution of pro-HJ was titrated with $MgCl_2$ over the range 0-20mM, with and without a 1μM solution of the complementary oligo. The FRET donor moieties are excited by a light source providing 476.5 nm radiation directed towards them and any output radiation from the FRET acceptor moieties is detected by a photomultiplier, using a Fluoromax Spectrofluorometer (Horiba Jobin Yvon Ltd., UK). When the aqueous buffer contained magnesium ions, the HJ structure adopted a "closed" configuration in

which FRET energy transfer to the FRET acceptor takes place resulting in an output radiation which can be detected. A significant conformational change is seen due to switching of the HJ population (background corrected maximum FRET ratio, where the FRET ratio is the ratio of the peak acceptor to peak donor emission intensities, of 0.7) compared with the control case without the complementary strand (background corrected maximum FRET ratio of 0.1). The switching transition is illustrated in Figure 13.

2. Alternative pro-HJ designs with internal dye labelling

Using the same procedure as described in Example 1, we have designed various alternative pro-HJ designs with fluorophore labels attached at different positions as follows: (a) one "internal" label at a fully formed double-stranded arm and one "external" label at a free end of the single-stranded pro-arm i.e. one at a non-terminal base internally of the oligonucleotide forming the pro-HJ, at the first arm with a dT loop , and one at a terminal base of said oligonucleotide, at the 5' end (b) two "internal" labels – one at each one of the fully formed double- stranded arms of the pro-HJ i.e. at both dT loops - see Figure 14.

With the addition of 1μM of complementary target oligonucleotide of (a) TGGGATTCGGACTATGCA and (b) GCATAGTGGATTGCA a significant change in switching characteristic is seen for these two structures. Ionic switching using 10mM $MgCl_2$ shows FRET ratios of (a) 0.47 and (b) 0.47, compared with (a) 0.15 and (b) 0.18 with no target molecule present.

3. Alternative dye positions

Using the same structures as described in Example 2, we have designed various alternative pro-HJ designs with fluorophore labels attached at different positions in the dT loop (Figure 15). The FAM donor dye is moved to every position of 1st dT loop in (a) and TAMRA acceptor dye is moved to every position of 2nd dT loop in (b) – see Figure 15.

All these structures show a significant change in switching characteristic is seen for these two structures with addition of 1μM of complementary target oligonucleotide. Ionic switching using 10mM $MgCl_2$ shows that the FRET ratios change corresponding to the differences in dye position over the range (a) 0.47, 0.36, 0.62, 0.59, 0.53 and (b) 0.47, 0.66, 0.73, 0.82, 0.60, compared with (a) 0.15, 0.16, 0.18, 0.18, 0.18 and (b) 0.18, 0.14, 0.13, 0.16, 0.13 with no target molecule present.

4. Alternative arm length

We have designed various alternative pro-HJ designs with increasing arm length, using the same structure as described in Example 3, (b), such that with longer arms the dyes are further apart in the 'open' configuration . A 1μM solution of these structures hybridised with 1μM complementary DNA oligonucleotide (TGCATAGTGGATTGGAGG) forms HJ-switch structures, which were then switched with the addition of 10mM $MgCl_2$.

Table 1. Sequence and features of extended arm bimolecular HJ's

SeqA:TGCAATCCTGAGCACATTTTTGTGCTCACCGAATCGGATTTTTCCGATTCGGACTATGCA

SeqB:TGCAATCCTGAGCACACGTGATTTTTCACGTGTGCTCACCGAATCGGAGGATTTTTCCTCCGATTCGGACTATGCA

SeqC:TGCAATCCTGAGCACACGTGATTTTTCACGTGTGCTCACCGAATCGGTCAGGATTTTTCCTGACCGATTCGGACTATGCA

SeqD:TGCAATCCTGAGCACACGTGAGTCCATTTTTGGACTCACGTGTGCTCACCGAATCGGTCAGGAGCATTTTTGCTCCTGACCGATTCG GACTATGCA

SeqE:TGCAATCCTGAGCACACGTGAGTCCATTTTTGGACTCACGTGTGCTCACCGAATCGGTCAGGAGCACATTTTTGTGCTCCTGACCGA TTCGGACTATGCA

Pro HJ	Length (nt)	Donor arm (bp)	Donor arm (Å)	Acceptor arm (bp)	Acceptor arm (Å)	FRET Ratio with target	FRET Ratio without targe
SeqA	60	8	27	10	34	0.32	0.12
SeqB	76	13	44	13	44	0.11	0.04
SeqC	80	13	44	15	51	0.11	0.04
SeqD	96	18	61	18	61	0.09	0.02
SeqE	100	18	61	20	68	0.09	0.01

Increasing arm length and the separation of the FRET dye pair alters the characteristic FRET background signals of the pro-HJ structures. The table above shows that the structures can all detect target oligos. With addition of 1μM of complementary target oligonucleotide a significant change in switching characteristic is seen during ionic switching using 10mM MgCl. This illustrates that increasing arm length is a tuneable factor in selecting optimum HJ switch performance. The optimum will depend on the application for which the switch is used, will vary for different macromolecular structures and may be determined by trial and error.

5. Forming a macromolecular switch with RNA target molecule

The procedure of Example 2 was repeated using the proHJ (a) and the RNA target oligo UGGGAUUCGGACUAUGCA in place of the DNA target oligo TGGGATTCGGACTATGCA.

With addition of 1μM of complementary target oligonucleotide of GCATAGTGGATTGCA a significant change in switching characteristic is seen. Ionic switching using 10mM MgCl shows FRET ratios of 0.48 compared with 0.19 when no target molecule is present. This illustrates the ability of the pro-HJ macromolecular structure probe to detect an RNA target with a sequence corresponding to that of the complementary DNA oligonucleotide.

6. Label-free detection of RNA at low target concentrations

The procedure of Example 5 was repeated using 10nM of pro-HJ structure (a) from example 2 with RNA target oligo UGGGAUUCGGACUAUGCA at a very low concentration (10 nM). With addition of complementary target RNA a significant change in switching characteristic is seen. Ionic switching using 10mM $MgCl_2$ shows FRET ratios of 0.53 compared with 0.18 with no target molecule present.

This illustrates that a clearly positive detection signal can be obtained at low level concentrations of the target oligonucleotide.

7. Label-free detection of RNA from complex mixture at low target concentration

To illustrate the ability to detect RNA molecules from a complex biological mixture the procedure of Example 6 was repeated using the RNA target oligo UGGGAUUCGGACUAUGCA in the presence of varying background concentrations of non-complementary murine renal total RNA. With addition of complementary target RNA a significant change in switching characteristic is seen. Ionic switching using 10mM $MgCl_2$ shows FRET ratios of 0.41 compared with 0.22 with no target molecule present.

This illustrates that a clearly positive detection signal is not subject to interference by the presence of other cellular RNA, thereby showing the effectiveness of the present invention in detecting RNA targets in real-life samples.

8. Single base discrimination of target

The binding and switching properties of several targets which differ in their sequence at a point which binds to the pro-HJ in the region of the HJ the branch point, were studied as follows. Target oligonucleotide with the following sequences were made by solid phase synthesis: (a) no mismatch TGGGATTCGGACTATGCA, (b) single base mismatch at the 11^{th} base position TGGGATTCGGGCTATGCA. . Fig 16A illustrates the alignment of these targets with the pro-HJ probe.

A gelFRET assay (Ramirez-Carrozzi & Kerpolla, 2001, Methods 25, 31-43) was carried out. Samples were annealed overnight at a ratio of 10 μM pro-HJ probe: 50 μM target. The annealed samples were then diluted ten fold in 5.0 mM $MgCl_2$ and 45 mM Tris/Borate pH 8.3 prior to loading on the gel. Gels were run at 4 °C for 90 min at 75 Volts. Gels were then scanned using a laser to excite at 488 nm and emission filters to detect at 520 nm (the range is 500 – 540 nm) and 580 nm (the range is 565 – 595 nm); the bandpass is 560 nm. The intensity of the signal obtained at 520 nm (represented by the intensity of the colour red in the original figure) was overlaid with the intensity of the signal obtained at 580 nm (represented as the intensity of the colour green in the original figure) to visualize the relative difference in FRET for each gel band. The colour of each gel band is indicated above the lane in figure 16B.

Both targets bind to the pro-HJ, as shown by the gel shift when compared with the no target lane - Figure 16 B 1. The resultant HJ structures switch to differing degrees in 5mM $MgCl_2$. The single nucleotide mismatch (b) has significantly reduced switching (RED) when compared with (a) the no mismatch structure (GREEN) i.e. the detection of single nucleotide differences in DNA targets, is possible

Figure 16 B 2 shows that these structures switch to the open conformation if the Mg^{2+} is removed by soaking in 0.1 mM ethylenediaminetetra(acetate) EDTA (RED). Figure 16 B 3 shows that the no mismatch structure switches back to the closed conformation (GREEN) if the $MgCl_2$ is reintroduced i.e. illustrates that the switchable biosensor is reversible.

When a 1μM solution of pro-HJ is annealed with (a) 1μM no mismatch target and (b) 1μM single nucleotide mismatch target and then titrated with $MgCl_2$ over the range 0-20mM, a

significant conformational change is seen in (a) due to switching of the HJ population (background corrected maximum FRET ratio of 0.8) compared with (b) the single base mismatch (background corrected maximum FRET ratio of 0.3). The control titration without any target has a background corrected maximum FRET ratio of 0.25. The switching transition is illustrated in Figure 16 C.

9. Confirmation of independence of target binding and ion-induced switching

The pro-HJ shown in Figure 4 was prepared as described in Example 1 and annealed overnight with/without the DNA target. A gelFRET assay (Ramirez-Carrozzi & Kerpolla, 2001, Methods 25, 31-43) is then used. In this procedure the HJ samples are loaded onto polyacrylamide gels (90 mM Tris/Borate, pH 8.3, 10 % acrylamide and either 0 or 5.0 mM $MgCl_2$) at 2.5 uM (-/+ 5.0 uM DNA or RNA target). Gels were run at 4 °C for 90 min at 75 Volts. Gels were then scanned using a laser to excite at 488 nm and emission filters to detect at 526 nm (the range is 500 – 540 nm) and 580 nm (the range is 565 – 595 nm); the bandpass is 560 nm. The intensity of the signal obtained at 526 nm (represented by the intensity of the color red in the original figure) was overlaid with the intensity of the signal obtained at 580 nm (represented as the intensity of the color green in the original figure) to visualize the relative difference in FRET for each gel band. The overall color of each gel band is indicated above the lane in the Figure, with GREEN meaning high levels of FRET and RED meaning low levels of FRET.

The DNA target binds to the pro-HJ (shown by the relative band positions of pro-HJ with and without targets in Figure 4). Switching of the HJ is induced by changing from 0 mM $MgCl_2$ (shown as RED in Figure 4) to 0.5mM $MgCl_2$ (shown as GREEN in Figure 4). This illustrates that target binding and ion-induced switching are independent processes.

10. Design and use of HJ probes to detect naturally-occurring nucleic acid sequences

HJ probes were designed to detect specific sequence of the mouse Ccl5 gene (Mm.284248) and specific sequence of the mouse Cxcl9 gene (Mm.766), in this case based on pro-HJ structures described in Example 1. The selected probe designs are shown in Table 2.

Table 2. Sequence of Ccl5 and Cxcl9 ProHJ probes

CCL5 Probe	TCTTGAACCCTGAGCACATTTT*T*GTGCTCACCGAATCGGAT<u>T</u>TT TCCGATTCGGACTTCTTC
CXCL9-Probe	AGCTACAGCCTGAGCACATTTT*T*GTGCTCACCGAATCGGAT<u>T</u>T TTCCGATTCGGACAACCAA

T=FAM deoxythymidine, <u>T</u>=TAMRA deoxythymidine

The DNA target binds to the pro-HJ (shown by the relative band positions of pro-HJ with and without targets in Figure 18). Switching of the HJ is induced in the presence of 5mM $MgCl_2$ (shown as RED in Figure 18). This example illustrates that HJ probes can be designed to detect naturally-occurring nucleic acid sequences.

11. Design and Use of HJ probes to detect naturally occurring SNP

Sequence analysis of a panel of SNPs present on the Affymetrix genotyping platform was performed to look for sequences which showed good homology to the target sequences of pro-HJ structures described in Example 1, particularly focusing on the sequence at the branch point of the HJ to detect SNP A-2131935 (www.affymetrix.com, ref SNP ID: rs12270338) discriminating between C and A alleles. The selected probe designs are shown in Table 3. Initial work to detect the A allele suggested that the HJ molecule switched to an alternate conformation, in the light of this the position of the FAM donor dye was switched to the 3' end of a 2 bp extended molecule to allow an increase in FRET signal upon switching. Figure 19 shows a schematic representation of the Mg^{2+} induced switching, shown by the arrows, of the two probes when detecting their respective targets.

Table 3. Sequence of SNP A-2131935 C and A ProHJ probes

Pro_A-2131935C	CAGAATCCTGAGCACATTTT*T*GTGCTCACCGAATCGGAT<u>T</u>TT TCCGATTCGGACTATGGC
Pro_A-2131935A	CAGAATCATGAGCACATTTTTGTGCTCACCGAATCGGATT<u>T</u> TTCCGATTCGGACTATGGCA<u>T</u>

T=FAM deoxythymidine, <u>T</u>=TAMRA deoxythmidine

The DNA target binds to the pro-HJ (shown by the relative band positions of pro-HJ with and without targets in Figure 20). Switching of the HJ is induced in the presence of 5mM $MgCl_2$ (shown as RED in Figure 20) is only observed with binding of a perfectly matched allele. This illustrates that HJs can be used to discriminate between single nucleotides in naturally occurring SNPs.

12. Detection of naturally-occuring SNPs in a clinical sample

Detection of naturally occurring SNPs in a clinical sample could be achieved after PCR amplification of the region encompassing the SNP and the use of a ProHJ to detect the amplified sample. For example, the region encompassing the SNP described in Example 11 could be amplified using the PCR primers described in Table 4 from clinical samples of genomic DNA.

Table 4. Sequence of PCR primers for amplification of region encompassing SNP _A-2131935

A-21_L2	GCACAAATATAGGAGGACAG
A-21_R2	CATTGAGTTTAAATGCCATA

These primers give rise to a 47bp PCR product which could subsequently be used as the target for SNP detection using proHJs Pro_A-2131935C and Pro_A-2131935A.

13. Use of 2-AP as Alternative Switching Detection System

A HJ has been constructed which has no donor or acceptor, but in which the adenine at the branch point has been exchanged for an analogue – 2-aminopurine (2AP) see figure 21 for details. In the open state, 2AP should fluoresce and on addition of Mg, when the HJ closes, the fluorescence should be quenched (due to base stacking) [*Biochemistry* **2001,** *40,* 946-956, Probing Structure and Dynamics of DNA with 2-Aminopurine: Effects of Local Environment on Fluorescence, Edward L. Rachofsky, Roman Osman, and J. B. Alexander Ross,] and the fluorescence will thus drop. Preliminary experiments (figure 4) showed this to be the case. This example shows how 2-AP may be used in a pro-HJ, instead of a FRET dye pair to detect HJ switching (see Figure 22).

14. Effects of single base mismatch throughout target sequence

To demonstrate the particular importance of the branch point sequence for specificity of detection we performed mutational analyses of positions both within and outside of the branchpoint. The FRET ratios of pro-HJs with the matched and mismatched targets were measured in solution at 5.0 mM MgCl2. As shown in Figure 23, mutation of the target at the branch point results in the most dramatic decrease in FRET ratio (and therefore switching). Mutations farther away from the branch point (positions 6, 7, 14, for example) do not influence switching, as compared to the matched target.

15. Switching on a surface

In order to demonstrate that a HJ probe-target complex can be switched on a surface, by adding different concentrations of magnesium ions, two identical arrays (Figure 24) were printed on a slide coated with glycidoxyproyl silane (Erie Superchip Epoxy obtained from Erie Scientific, New Hampshire, USA) comprising multiple spots of HJ probe-target complexes. The HJs are coupled to the slide via the formation of a covalent bond. Amine-epoxide coupling was chosen over thiol-mediated or biotin-streptavidin coupling by testing the switch characteristicis of the HJ when a functional group was added to either the 3' or 5' end. We found that adding an amine function had the least effect on the HJ's ability to switch. The sequence of the oligo was as follows, reading from the 5' end to the 3' end:

TGCAATCCTGAGCACATTTT1GTGCTCACCGAATCGGAT2TTTCCGATTCGGACT ATGCA

where the [3'] is modified with an NH2 moiety having a six-carbon linker and where 1 = denotes a donor-labelled deoxythymidine and 2 denotes an acceptor labeled deoxythymidine.

After blocking with ethanolamine, the arrays were covered with separate lifter slips and washed with solutions as indicated below. All solutions contained the target to ensure stablility of the HJ complex (1μM).

Washes were carried out in the following order:

	Subarray 1	Subarray 2
Wash 1	No Mg	5mM Mg
Wash 2	5mM Mg	No Mg
Wash 3	No Mg	5mM Mg
Wash 4	5mM Mg	No Mg
Wash 5	No Mg	5mM Mg

After each wash, the slides were scanned in the Tecan Scanner using the blue laser and simultaneously measuring emission at 530 and 580 nm. Figure 25 shows the change in FRET ratio with ion buffer exchange – the FRET ratio was calculated as the ratio between emission intensities: I_{580}/I_{530}. This example illustrates the ability to form proHJ + target complexes with the probe immobilized on a surface which can switch based on ionic conditions.

16. Electrochemical Control of HJ

Experiments were performed to induce HJ switching by the addition and removal of Zn^{2+} switching ions into solution through electrochemical oxidation (stripping) and rereduction of zinc, to establish the principle of electrochemical control. It was found that Zn reversible plating and stripping at a zinc wire electrode was inhibited by the formation of an insulating zinc oxide surface layer in aqueous solution, and reversible quantitative electrochemical stripping and plating could not be achieved in aerated solution due to chemical oxidation of zinc by oxygen in aerated solution (corrosion) [Ferapontova et al, Electrochem. Comm., **9** (2007) 303-9]. These effects were overcome by using a Zinc phosphate and Nafion® modified Zn wire [for procedure, see Heller, Anal. Bioanal. Chem., **385** (2006) 469]. This was then used as a working electrode (WE) to electrochemically deposit Zn from solutions containing 1 mM Zn^{2+} (added as $ZnSO_4$) in 20 mM Tris-HCl, pH 7.5 by holding the electrode potential at -1.2 V with respect to a home-made Ag/AgCl electrode reference electrode to reduce the Zn^{2+} ions, with a Pt wire counter electrode (CE). Deposition times were varied from 30 min to 1 h to vary the amount of Zn^{2+} removed from solution. After electrochemistry, the optical absorption of the solutions after the addition of 0.1-0.5 mM PAR (4-(2-pyridylazo) resorcinol – a stoichiometric complexant with Zn^{2+}) was recorded, and these data compared with equivalent data for the absorbance of 0.1-0.5 mM PAR with solutions produced by the chemical addition of $ZnSO_4$ to give solutions containing 0.2, 0.5 and 1 mM Zn^{2+} in 20 mM Tris-HCl, pH 7.5. Comparison of these data showed that up to 75% of the Zn^{2+} ions could be extracted electrochemically on this timescale from the working solutions, with a good reproducibility between different experiments.

A solution of HJ was prepared by annealing the following four strand sequences:

Oligo 1	*GGCA*FTGCAATCCTGAGCACA*TAGA* (5 μM)
Oligo 2	*TCTA*TGTGCTCACCGAATCGGA*CCAG* (10 μM)
Oligo 3	*CTGG*TCCGATTCGGACTATGCA*GCAA* (10 μM))
Oligo 4	*TTGC*RTGCATAGTGATTGCA*TGCC* (10 μM)

F is the donor Carboxyfluorescein (known commercially as FAM™) on oligonucleotide 1 and **R** is the acceptor Carboxytetramethylrhodamine (known as TAMRA™) on oligonucleotide 4. In this case, internal dyes were used to maintain dye position relative to the branch point. The HJ was assembled in a solution of 20 mM Tris/TrisH^+Cl^- (pH 7.5) buffer solution containing NaCl (50 mM) and $MgCl_2$ (5 mM), by heating to 80°C for 30 minutes and then allowing the solution to cool slowly in a water bath to room temperature. A ratio of 1:2:2:2 was chosen to ensure full incorporation of donor strand into the fully assembled 4-way junctions. The final step in the sample preparation was to buffer exchange twice (Microspin G25 ion exchange columns (Amersham Biosciences)) to removing the Mg^{2+} ions and produce HJ solutions (concentration 1 μM after dilution) in 20mM Tris/TrisH^+ Cl^- buffer (pH 7.5) as required for ion titration studies. 100 μM tetrasodium ethylenediamine(tetraacetate) Na_4EDTA was then added to the solution to titrate any residual Mg^{2+} ions and produce HJ exclusively in the open form.

The same electrode configuration as above was then used to sequentially add and remove Zn^{2+} to and from this solution of HJ (1μM) and 20 mM Tris-HCl, pH 7.5, through electrochemical oxidation (at -1.0 V) and reduction (at -1.2 V) at the WE. The steady-state fluorescence emission of the HJ was measured using a fixed excitation wavelength of 476.5 nm, and measuring the peak donor and acceptor emission at 518 nm and 578 nm respectively. The corresponding FRET ratio observed as a function of oxidation charge (+ charge values, corresponding to increasing Zn^{2+} concentration) and reduction charge (- charge values, corresponding to decreasing Zn^{2+} concentration) are shown in Figure 26. These results show a correlation between the proportion of closed MHJ (given by the magnitude of the FRET ratio) with the amount of Zn^{2+} electrochemically inserted into or removed from the solution (as measured by the sign and magnitude of charge passed). This example demonstrates the principle of reversible electrochemical control of HJ configurations, which can be readily extended to pro-HJ complexes.

17. Dye Location Design Considerations for Probe Design

To examine any effect of dye position on sequence specificity, we compared the switching properties of two different pro-HJ constructs with mismatched targets. In both pro-HJs, the donor is contained on an internal thymidine; in PD03, the acceptor is contained on the 3'end but in PD09, the acceptor is attached to an internal thymidine (see Figure 27). Mutations at the branch point have a significant effect on the FRET ratio in both cases, as expected. However, the pro-HJ with the acceptor at the 3'end (PD03) is sensitive to mutations in the target that are in proximitiy to the acceptor (even though they are not close to the branch point). This is not an issue for the probe with the internal acceptor (PD09).

This example illustrates the sensitive affect of base mismatches on FRET signaling. The design PD09 may be preferred in applications where a single area of precise specific sequence detection is required such as SNP detection. The design PD03 may be preferred if general sequence specification is required such as in transcript detection applications.

18. Use of lifetime measurement to detect HJ position

As an alternative to using the fluorescent intensity and emission wavelength to perform detection, it is possible to use time-resolved fluorescence measurements. Fluorescence lifetime measurements have general advantages over intensity based assays in that the fluorescence lifetime of molecules is independent of local fluorophore concentration and local excitation light intensity. In cases where FRET occurs, the decay of the excited state population is rapid and time domain data can be used to determine the quantitative separation between fluorescent species using the nonlinear dependence of the FRET rate constant on separation.

In this example, samples based on the DNA probe: (5-TGCATAGTGGATTGCATTTTTGCAATCCTGAGCACATTTTTGTGCTCACCGAATCCCA-3') were synthesised (Eurogentec) with Tetramethylrhodamine (TAMRA) attached at the 5' end and Carboxyfluorescein (FAM) attached at a thymidine (19 nucleotides from the 5' end). Probe variants were made with and without the FAM acceptor fluorophores. Target variants were made with the base at point 11 modified with a C base (11C) and a G base (11G) (see Figure 28). The probes (10 μM) were assembled in the presence of the target DNA oligonucleotide (purchased from Eurogentec) in 20 mM Tris/HCl (pH 7.5), 10 mM

MgCl2 at five fold excess of target. Samples were incubated in a water bath at 80C for 30 min, followed by a slow temperature decrease to room temperature. Time-resolved fluorescence data were collected by donor excitation from a mode locked, frequency doubled Coherent MIRA 900-F to produce vertically polarised, 200 fs pulses of wavelength 450 nm. Emission was collected in an Edinburgh Instruments FL-920 spectrometer with the monochoromator set at 517 nm, the peak of the donor emission. The emission polariser was set at 54.7 to the vertical to avoid anisotropy effects. All data were collected to a peak of 10,000 counts with a delay window of 20 ns and 4096 channels.

Figure 29 shows time-resolved FRET decays for a donor only molecule, plotted alongside data for a switch incorporating target 11C and 11G. This clearly shows that sufficient resolution is achieved to allow single base changes around the branch point to be resolved.

19. Use of multiple state switch systems to detect HJ conformers.

In order to assess whether mutations in the target sequence influence the conformer presence of the HJ (and whether this can be detected), different acceptor dyes were introduced into the HJ, such that either of the closed conformer could be detected and would produce a different output signal, since the acceptors differ in their emission wavelengths (see schematic in Figure 30). In this example, the "target" strand was strand 3 (the strand with no donor/acceptor moiety), and the mutation examined was at the branchpoint ("mismatched" is a G at position 11 instead of an A). As can be seen, the mismatched target has a decreased output signal for the TAMRA acceptor but an increased signal for the cy5 acceptor, compared to matched target, which is evidence for a different distribution of the two closed conformers.

This example effectively illustrates the ability to form a 3-state switch (closed, open I and open II) which adopts different states depending on the target molecule bound to the pro-HJ. As can be seen from the figure, the match and mismatch case can be discriminated either by a change in one acceptor dye signal, or the other or both. This gives the opportunity to more precisely discriminate the specific match/mismatch cases from each other and from non-specific binding.

Alternatively, if the acceptor dyes are both the same type, then the pro-HJ probe will give a signal output in the closed position based on the combined dye signal, regardless of the

conformer. This could be used if probe design constraints (i.e. designing a probe HJ to both form a switch and to achieve specific complementary binding) result in the need the use a probe with a, say 50:50 conformer preference characteristic rather than a 90:10 ratio. In this application, the application does not care which conformer is preferred.

20. Temperature-dependence of probe-target assembly.

In order to assess any temperature requirement for probe-target assembly, the switching parameters of mismatched targets were compared for samples that had been annealed at 80 C for 5 min in 20 mM Tris/Cl pH 7.5 (cooled to room temperature over 30 minutes) versus samples that were annealed at room temperature (< 2 min prior to FRET measurement). These samples were analyzed with gelFRET and there was no appreciable difference in FRET ratio for the two different annealing conditions (see Figure 31, n = 1 for RT annealing and n = 3 for 80 C annealing).

21. Optimal conditions for maximal discrimination of mismatched target

Buffer conditions and probe concentrations were examined in order maximize discrimination of target mismatch sequences. Using gelFRET, as described in Example 8, at 5.0 mM MgCl2 (1 uM probe, 5 uM target – gray bars in Figure 19), the branchpoint mismatch targets are fully bound and result in 0-4 fold decreased switching. In comparison, under conditions were mismatched target sequences are less optimally bound (samples prepared by mixing 50 nM probe with100 nM target at 20 mM Tris pH 7.6 and 0.1 mM MgCl2 at room temperature for 10 minutes prior to measurement – black bars in Figure 32), solution measurements demonstrate up to 30-fold discrimination between matched and mismatched targets. Solution measurements were conducted as described in Example 1. The data are normalized in the figure to maximal signal (matched target) compared to minimal signal (no target). The unnormalized FRET ratios for probe in the absence of target and in the presence of matched target were 0.46 and 1.23 (5.0 mM $MgCl_2$) and 0.42 and 0.91 (0.1 mM $MgCl_2$), respectively.

More stringent binding conditions were examined (10 nM probe: 20 nM target, 0.1 mM MgCl2), but resulted in a diminished FRET ratio of matched target (0.6 with target compared to 0.4 without target in MgCl2 – only 0.2 FRET difference), and would therefore decrease signal/noise and sensitivity required for discrimination. In addition, at such low concentrations, time can influence the detection signal, since binding is much slower and also signal to noise is higher (see Figure 33).

CLAIMS

1. A method for detecting a target analyte in a sample, which method includes the steps of: providing a pro-macromolecular structure switch wherein said pro-switch has an analyte binding site formed and arranged for binding said analyte with high specificity so as to form together therewith, a macromolecular switch, comprising: an oligomeric macromolecular structure having at least one selective ion coordination site, and arms flippable between a plurality of narrowly defined discretely different conformations of said oligomeric macromolecular structure corresponding to different ion binding conditions at said at least one selective ion coordination site, at least in the absence of any other binding to said oligomeric macromolecular structure which would interfere with flipping between said different conformations in response to a change between said different ion binding conditions, wherein said discretely different conformations are capable of providing, in use, with an output signal reading system, characteristic output signals, and wherein said pro-macromolecular structure switch has a substantially different switching functionality from that of said oligomeric macromolecular structure and is substantially incapable of providing, in use, with said output signal reading system, said characteristic output signals;
contacting said pro-switch with a said sample under binding conditions, so as to form a said macromolecular structure switch with any said target analyte present;
providing a said output signal reading system;
applying an external input signal to said at least one input device so as to change the ion binding conditions at said ion binding site so that said macromolecular structure switch is flipped from a first said conformation to a second said conformation; and
reading out any change in said output signal.

2. A method as claimed in claim 1 wherein said ion binding conditions are changed so as to increase the concentration of a switching ion at said ion binding site

3. A method as claimed in claim 1 or claim 2 wherein is used a switching ion selected from Zn^{2+}, Ca^{2+}, and Mg^{2+}.

4. A method as claimed in any one of claims 1 to 3 which includes the further steps of:
removing said external input signal; and
reading out any further change in said output signal.

5. A method as claimed in claim 4 which includes the further step of:
reading out a said output signal before contacting said pro-switch with a said sample; and
comparing all the output signals obtained with a truth table comprising:

Before target addition	Post target addition	When external signal applied	When external signal removed	Interpretation
Open	Open	Closed	Open	Target presence confirmed

6. A method as claimed in claim 4 which includes the further step of:
comparing all the output signals obtained with a truth table comprising:

Before target addition	Post target addition	When external signal applied	When external signal removed	Interpretation
Open	Open	Open	Open	Target not present
Closed	Any	Any	Any	Device failed
Open	Closed	Any	Any	Non-specific binding
Open	Open	Closed	Closed	Non-specific binding
Open	Open	Closed	Open	Target presence confirmed

7. A method of detecting an SNP using a method as claimed in any one of claims 1 to 6, wherein said analyte binding site is formed and arranged, so that said SNP binds to the pro-HJ switch at the junction region of said HJ switch.

8. A method of detecting RNA splicing using a method as claimed in any one of claims 1 to 6, wherein said analyte binding site is formed and arranged, so that it corresponds to a target sequence that spans the junction between two exons.

9. A method of detecting genetic lesions resulting from insertions in DNA sequences using a method as claimed in any one of claims 1 to 6, wherein said analyte binding site is formed and arranged, so that it corresponds to a target sequence that spans the junction across the

insertion site, preferably with the branch point aligned with the boundary across the insertion site

10. A method of detecting genetic lesions resulting from deletions in DNA sequences using a method as claimed in any one of claims 1 to 6, wherein said analyte binding site is formed and arranged, so that it corresponds to a target sequence that spans the junction between deletion break points.

11. A pro-macromolecular structure switch suitable for use in detecting an analyte, wherein said pro-switch has an analyte binding site formed and arranged for binding said analyte with high specificity so as to form together therewith,
a macromolecular switch, comprising: an oligomeric macromolecular structure having at least one selective ion coordination site, and arms flippable between a plurality of narrowly defined discretely different conformations of said oligomeric macromolecular structure corresponding to different ion binding conditions at said at least one selective ion coordination site, at least in the absence of any other binding to said oligomeric macromolecular structure which would interfere with flipping between said different conformations in response to a change between said different ion binding conditions, wherein said discretely different conformations are capable of providing, in use, with an output signal reading system, characteristic output signals, and wherein said pro-macromolecular structure switch has a substantially different switching functionality from that of said oligomeric macromolecular structure and is substantially incapable of providing, in use, with said output signal reading system, said characteristic output signals.

12. A pro-macromolecular structure switch as claimed in claim 11 wherein said oligomeric macromolecular structure comprises at least one of an oligonucleotide, an oligopeptide, and an oligosaccharide, macromolecular structure.

13. A pro-macromolecular structure switch as claimed in claim 11 or claim 12 wherein said oligomeric macromolecular structure comprises not more than 500 repeating units selected from nucleotide, amino acid, and saccharide, units.

14. A pro-macromolecular structure switch as claimed in any one of claims 11 to 13 wherein said pro-oligomeric macromolecular structure is provided with an output signalling device

15. A pro-macromolecular structure switch as claimed in any one of claims 11 to 14 wherein said output signalling device comprises a separation sensitive output signalling device

16. A pro-macromolecular structure switch as claimed in any one of claims 11 to 15 wherein said separation sensitive output signalling device comprises first and second components disposed on said pro-oligomeric macromolecular structure, remote from said selective ion coordination site thereof

17. A pro-macromolecular structure switch as claimed in any one of claims 11 to 16 wherein said separation sensitive output signalling device comprises a FRET system

18. A pro-macromolecular structure switch as claimed in any one of claims 11 to 17 wherein said pro-oligomeric macromolecular structure is anchored to a substrate

19. A pro-macromolecular structure switch as claimed in any one of claims 11 to 18 wherein said selective ion coordination site comprises a selective ion coordination pocket

20. A pro-macromolecular structure switch as claimed in any one of claims 11 to 19 wherein said oligomeric macromolecular structure comprises an HJ

21. A pro-macromolecular structure switch as claimed in any one of claims 11 to 20 wherein said pro-HJ consists essentially of an unimolecular oligonucleotide

22. A pro-macromolecular structure switch as claimed in any one of claims 11 to 21 wherein said pro-HJ consists essentially of a plurality of oligonucleotides covalently cross-linked so as to form an unimolecular entity

23. A pro-macromolecular structure switch as claimed in any one of claims 11 to 22 wherein said analyte binding site comprises single-stranded pro-arms, each having a length of at least 8 nucleotide base units

24. A pro-macromolecular structure switch as claimed in any one of claims 11 to 23 wherein said analyte binding site comprises single-stranded pro-arms, each having a length of from 10 to 60 nucleotide base units

25. A pro-HJ oligonucleotide suitable for use as a pro-HJ structure for binding with a target oligonucleotide, said pro-HJ oligonucleotide comprising: first and second, target binding, portions, each having a base sequence such that together they are complementary to the base sequence of said target oligonucleotide, whilst being substantially non-self-complementary; third and fourth portions separated by a fifth, linkage, portion, said third and fourth portions being complementary to each other when folded back across each other about said fifth linkage portion; and sixth and seventh portions separated by a eighth, linkage, portion, said sixth and seventh portions being complementary to each other when folded back across each other about said eighth linkage portion, said third to eighth portions extending serially between said first and second portions.

26. A pro-HJ oligonucleotide as claimed in claim 25 wherein said first and second portions are respective terminal portions of the pro-HJ oligonucleotide.

27. A pro-HJ oligonucleotide as claimed in claim 25 or claim 26 for use in detecting an SNP, wherein said analyte binding site is formed and arranged, so that said SNP binds to the pro-HJ switch at the junction region of said HJ switch.

28. A pro-HJ oligonucleotide as claimed in claim 25 or claim 26 for use in detecting RNA splicing, wherein said analyte binding site is formed and arranged, so that it corresponds to a target sequence that spans the junction between two exons, preferably with the branch point aligned with the boundary between the two exons.

29. A pro-HJ oligonucleotide as claimed in claim 25 or claim 26 for use in detecting genetic lesions resulting from insertions in DNA sequences, wherein said analyte binding site is formed and arranged, so that it corresponds to a target sequence that spans the junction across the insertion site, preferably with the branch point aligned with the boundary across the insertion site

30. A pro-HJ oligonucleotide as claimed in claim 25 or claim 26 for use in detecting genetic lesions resulting from deletions in DNA sequences, wherein said analyte binding site is formed and arranged, so that it corresponds to a target sequence that spans the junction between deletion break points, preferably with the branch point aligned with the boundary across the deletion break points

31. A set of pro-macromolecular structure switches according to any one of claims 11 to 26 for use in transcript profiling, comprising a multiplicity of pro-HJs with analyte binding sites which correspond to a multiplicity of different target sequences.

Figure 1

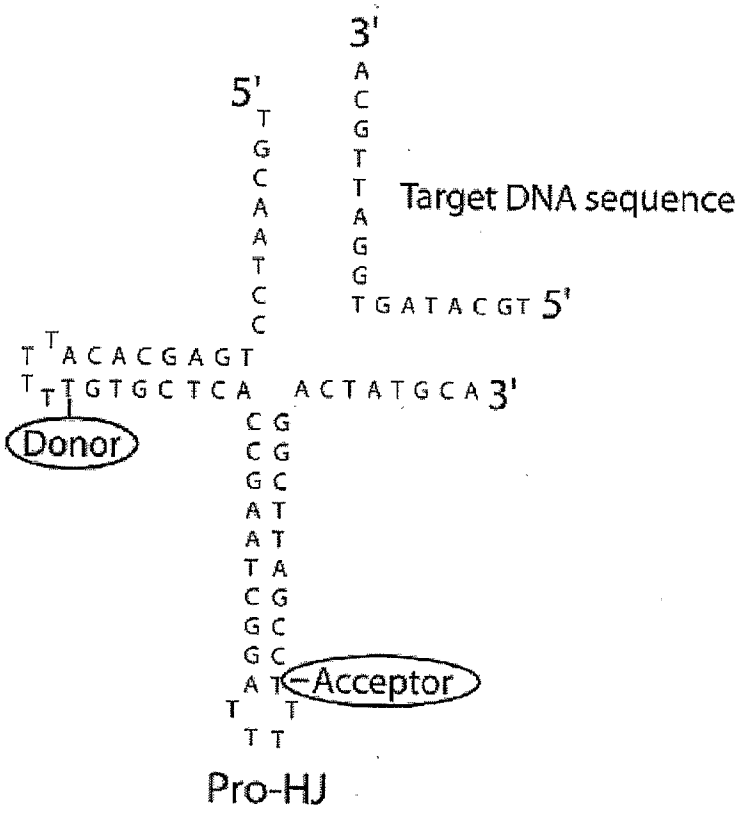


Figure 2

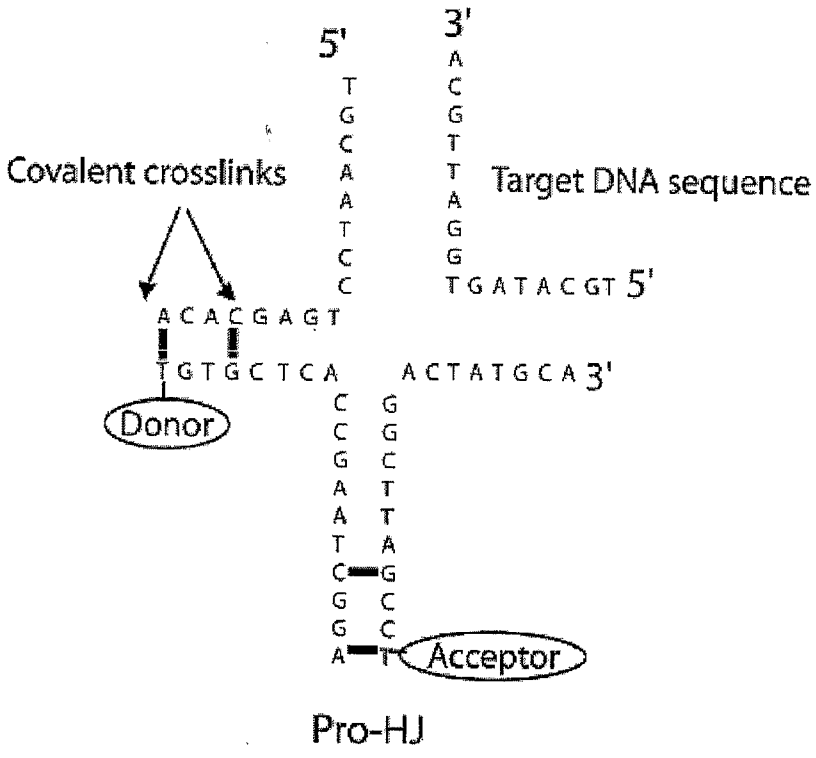


Figure 3

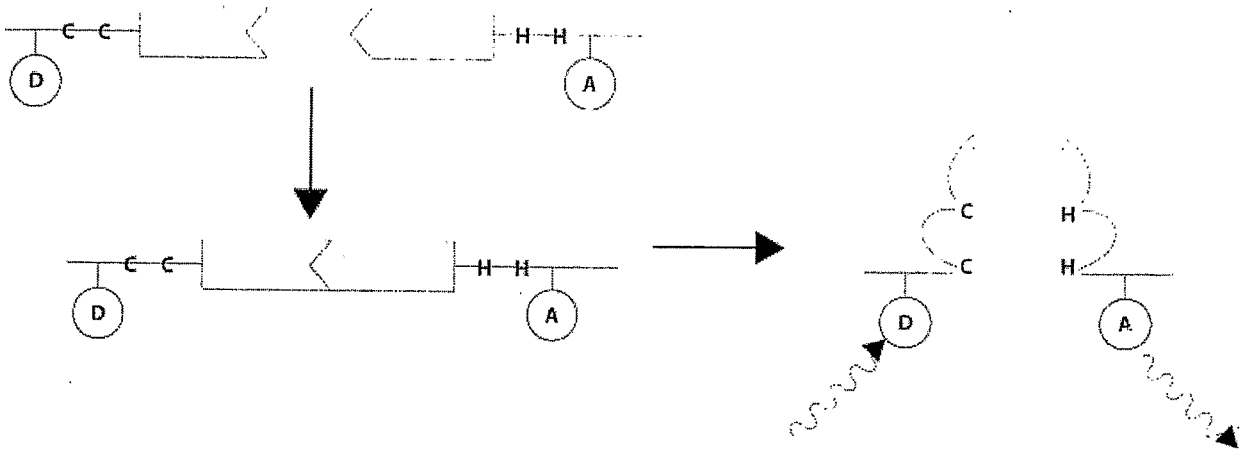


Figure 4

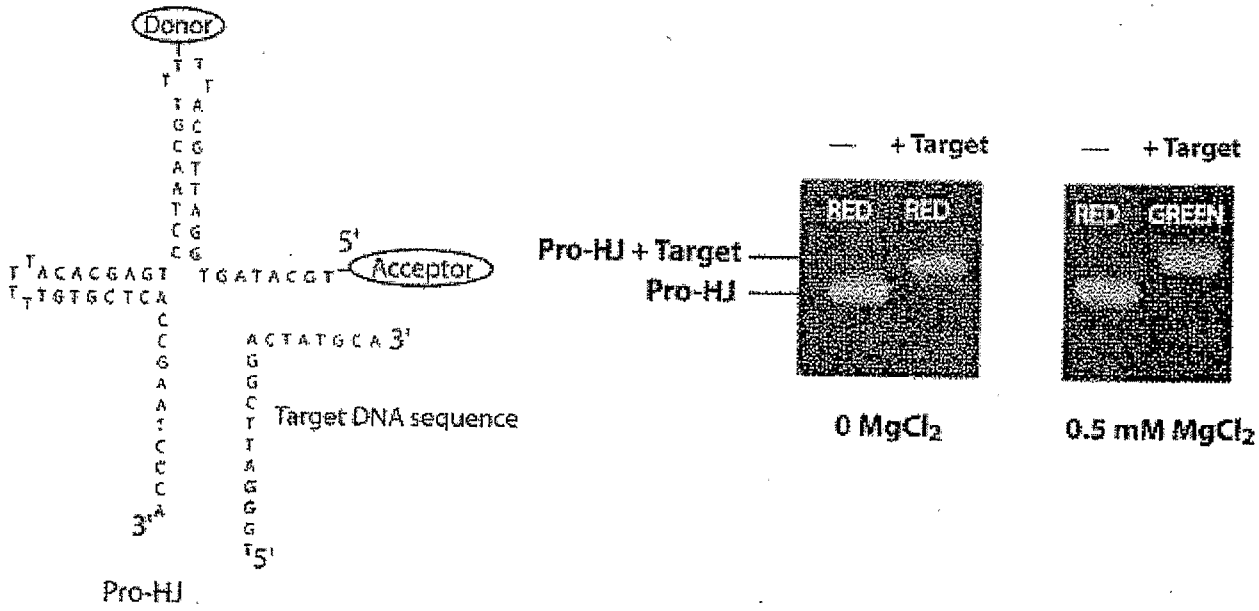


Figure 5

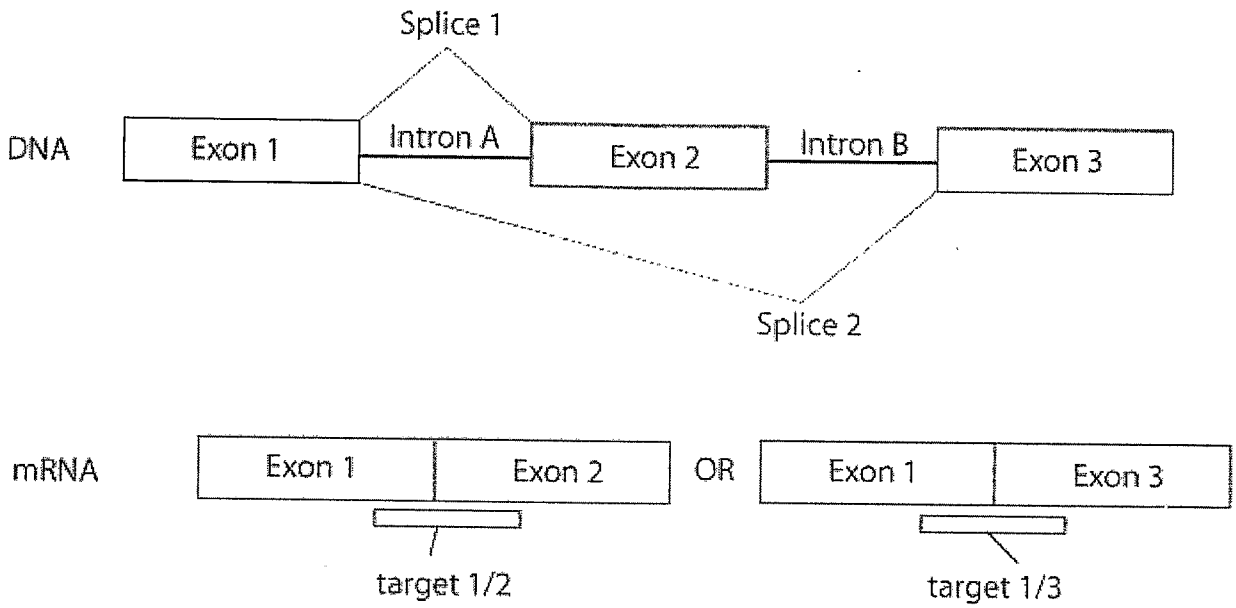


Figure 6

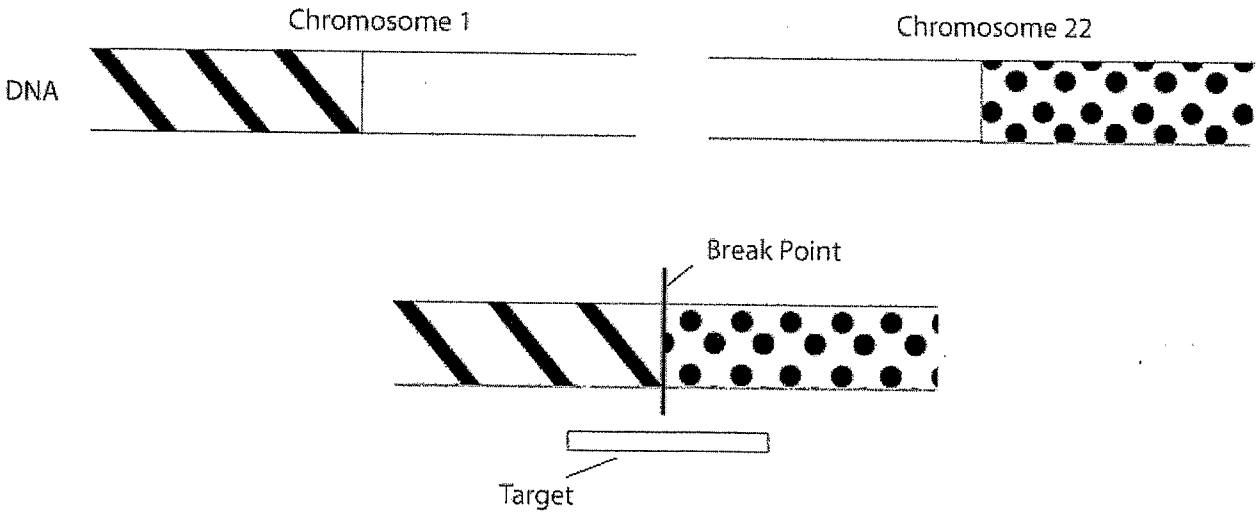


Figure 7

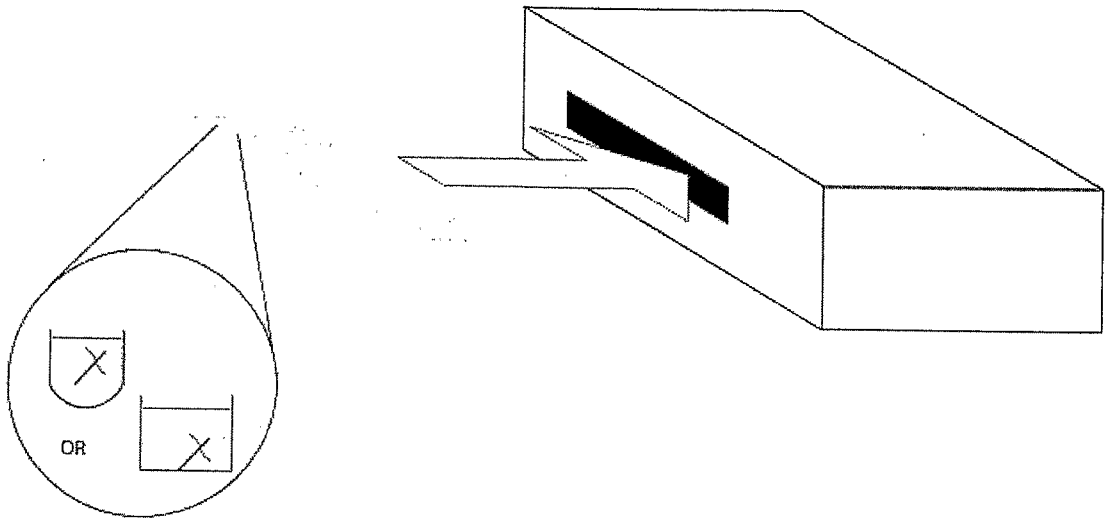


Figure 8

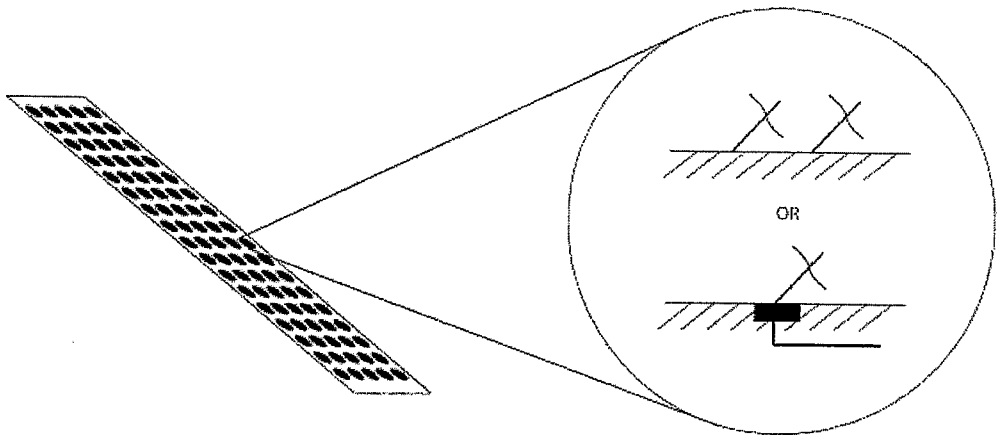


Figure 9

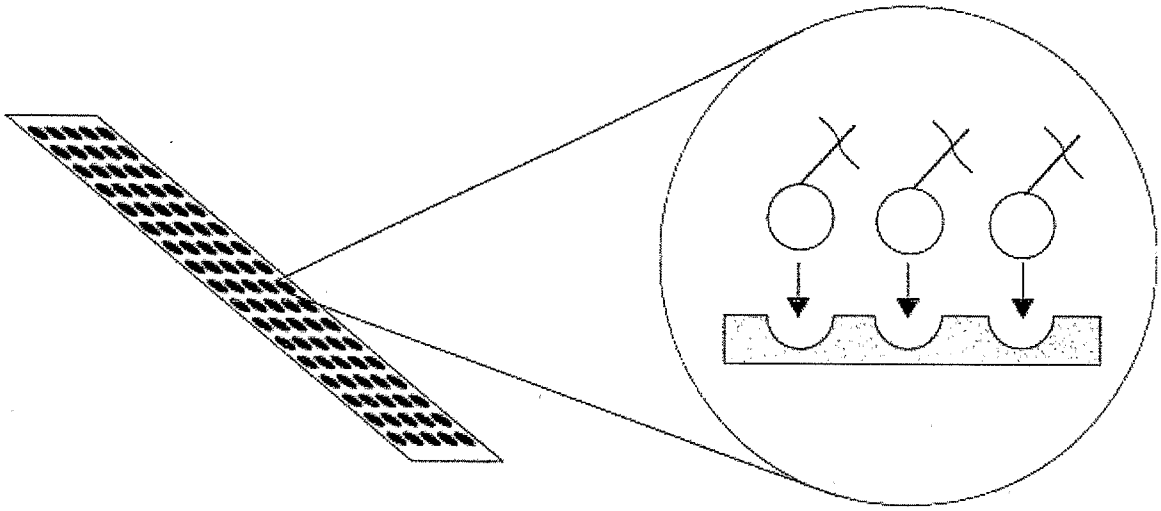


Figure 10

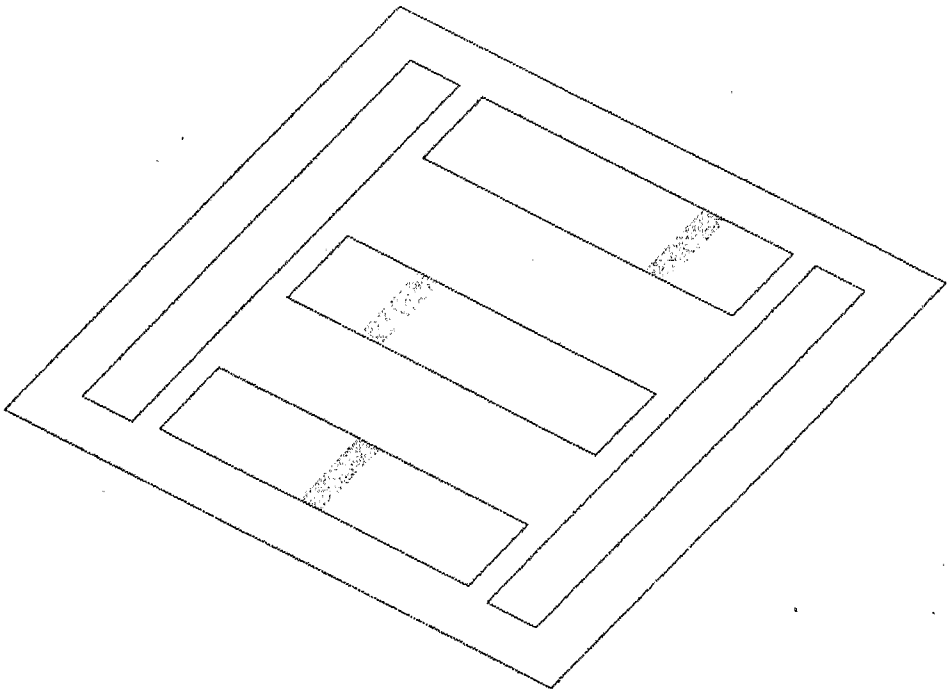


Figure 11

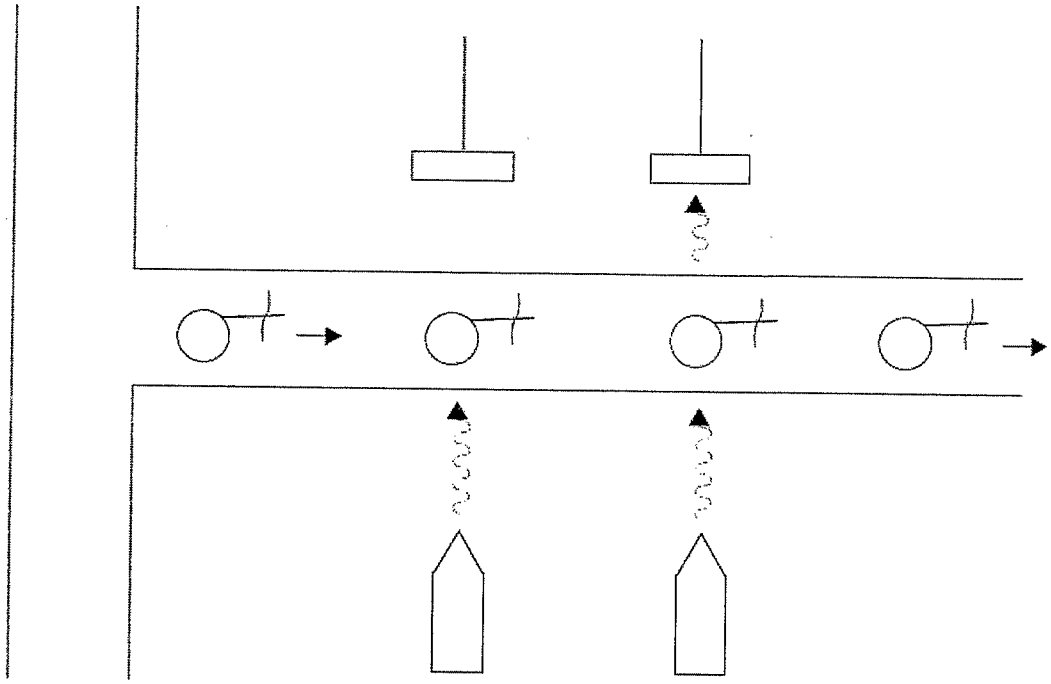


Figure 12

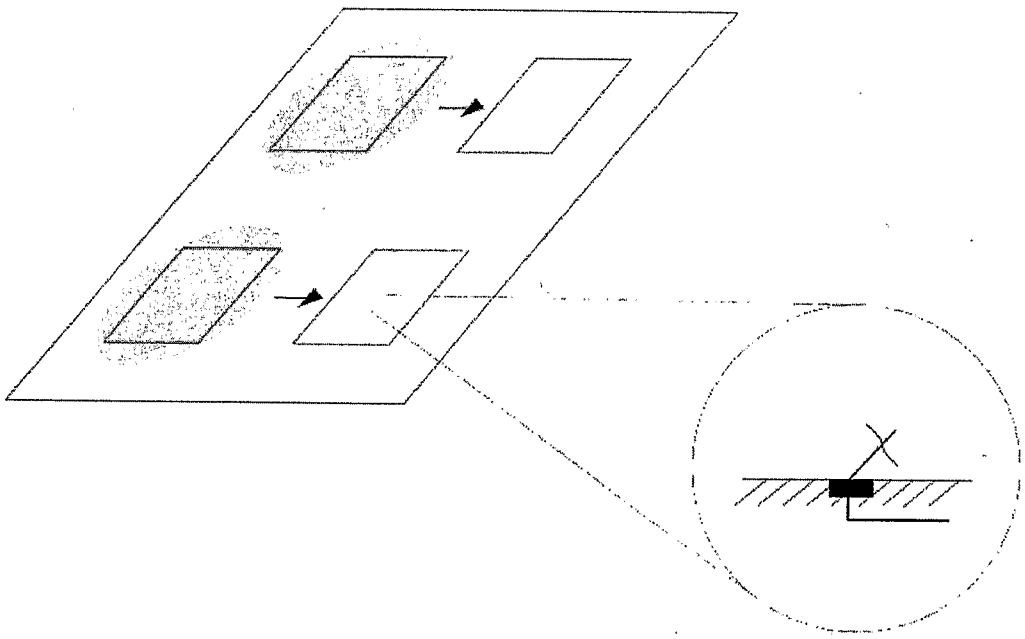


Figure 13

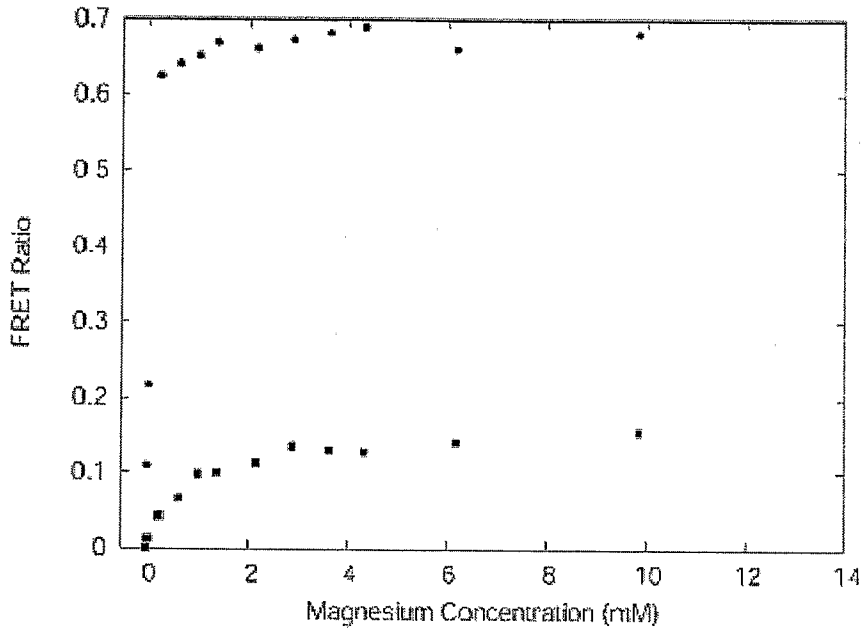
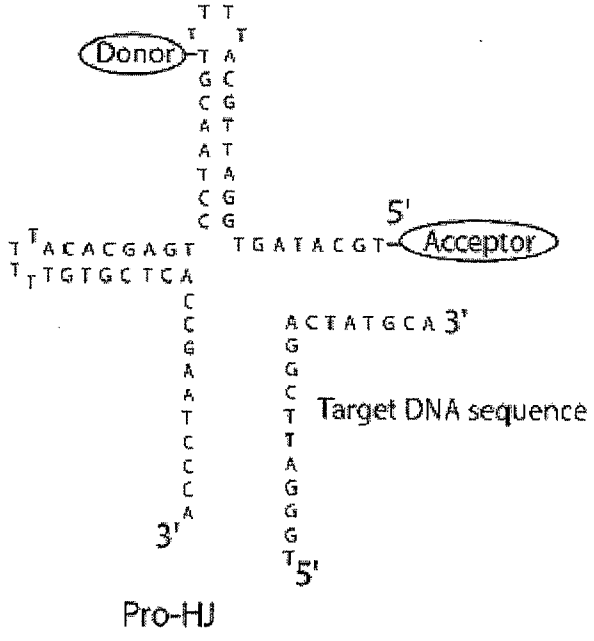


Figure 14

(A)



(B)

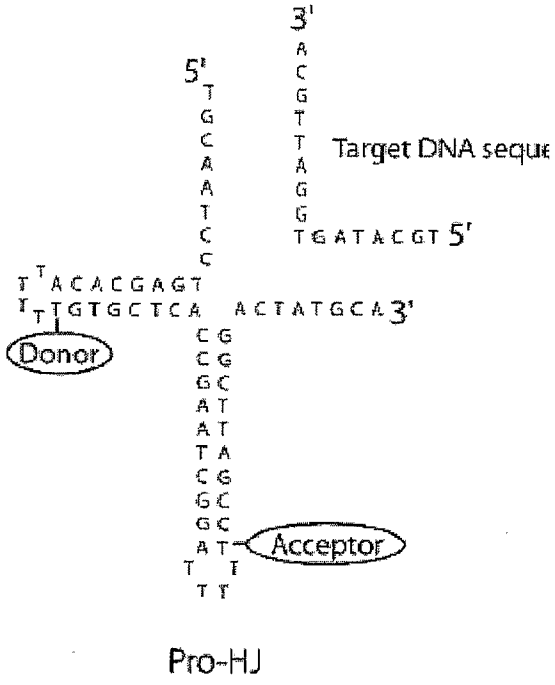
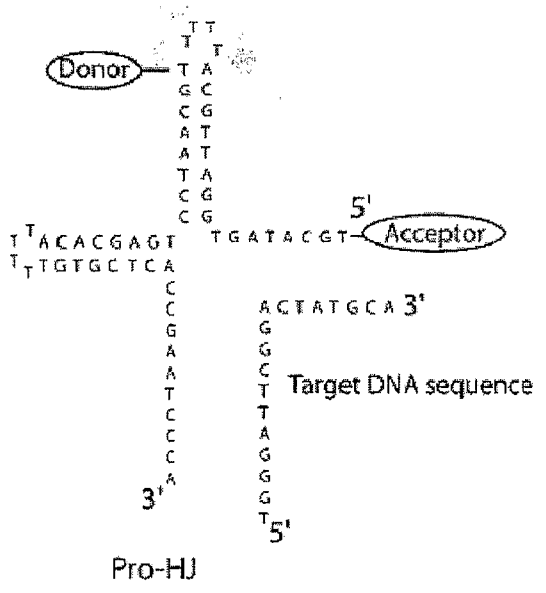
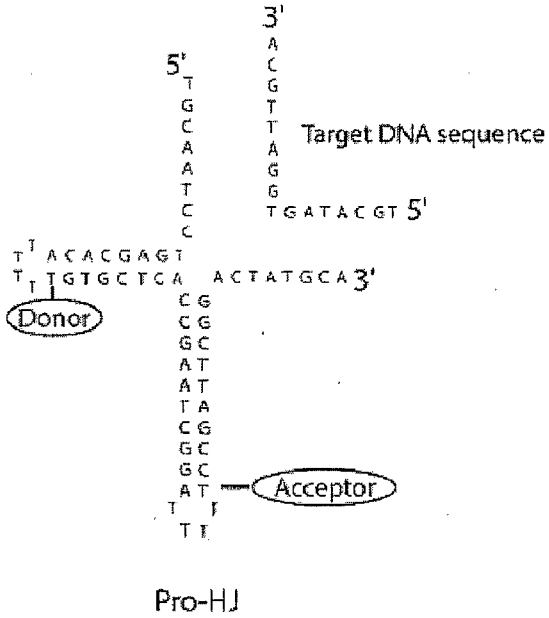


Figure 15

(A)


Donor
Acceptor
5'
3'
Target DNA sequence
Pro-HJ

(B)


3'
5'
Target DNA sequence
Donor
Acceptor
Pro-HJ

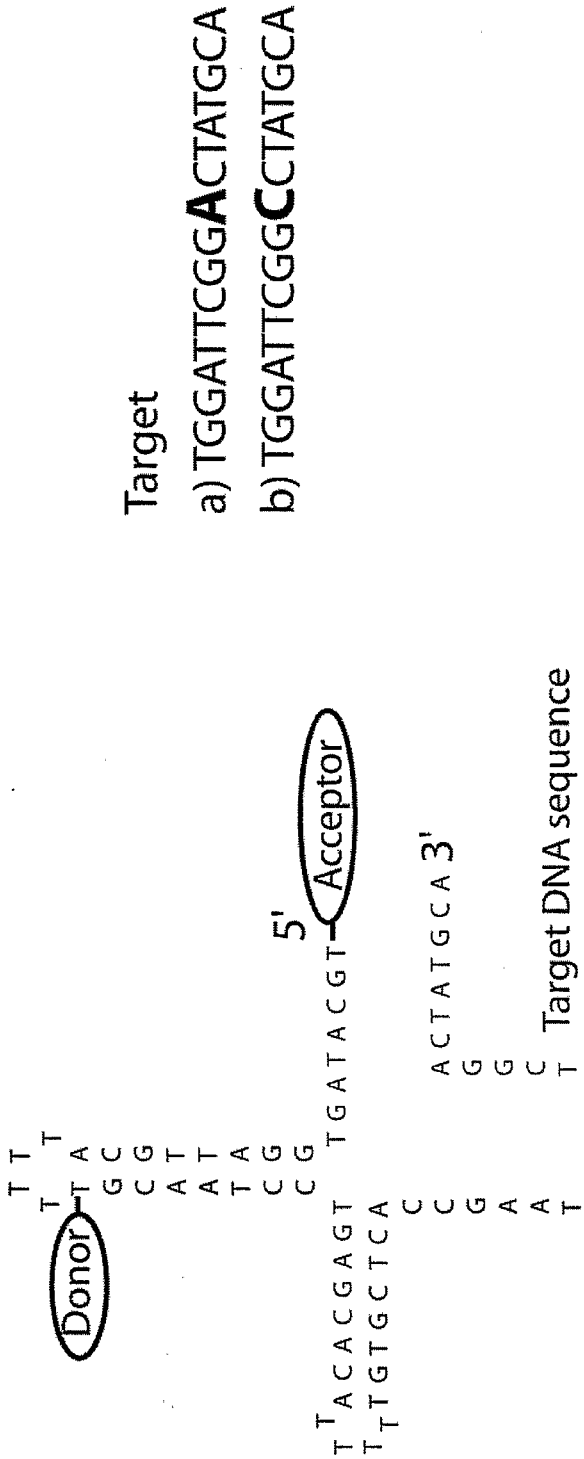
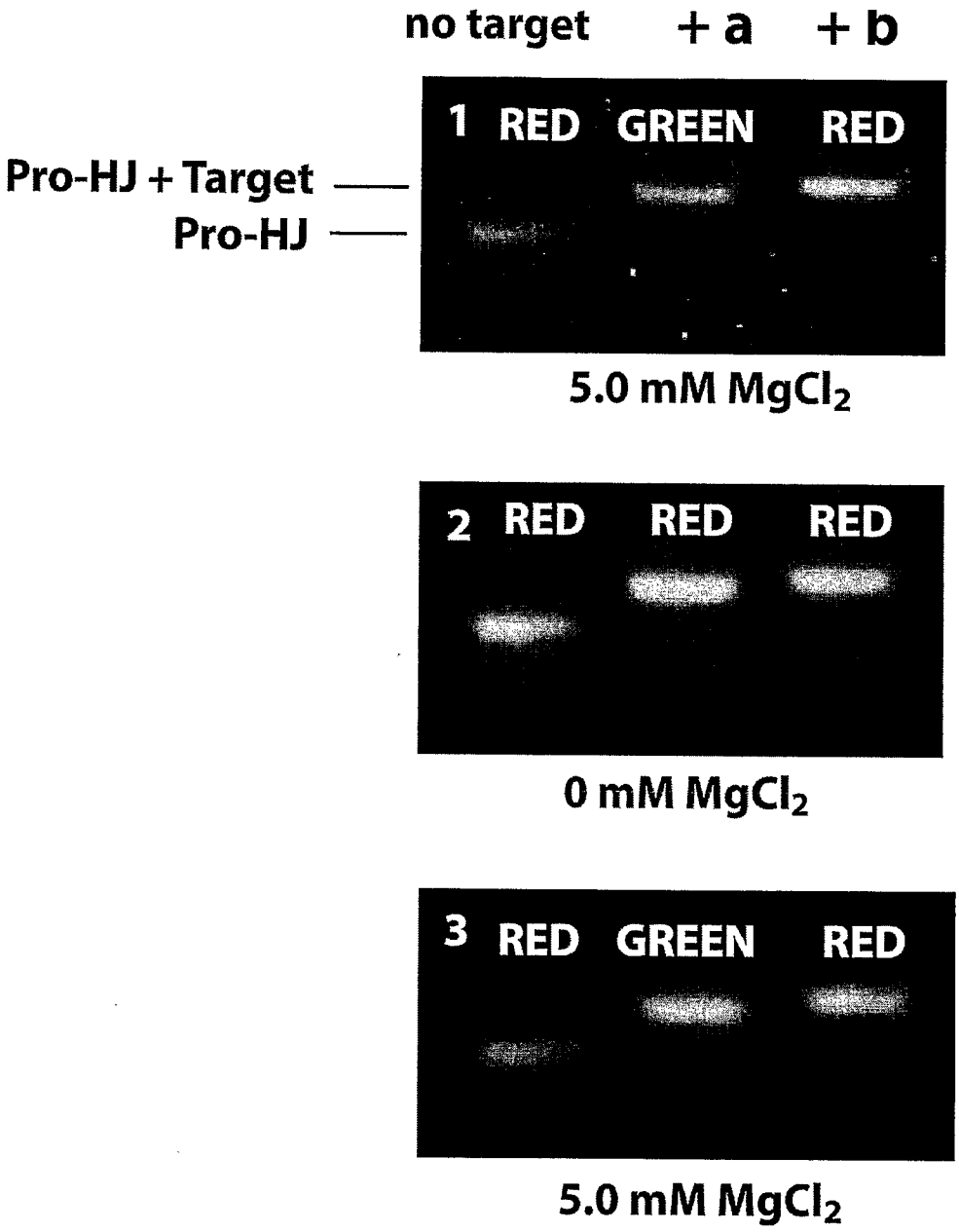

Target
a) TGGATTCGGACTATGCA
b) TGGATTCGGCCTATGCA
Donor
Acceptor
5'
3'
Pro-HJ
5'
3'
Target DNA sequence

Figure 16a

Figure 16b


no target
+ a
+ b
Pro-HJ + Target
Pro-HJ
1 RED GREEN RED
5.0 mM MgCl2
2 RED RED RED
0 mM MgCl2
3 RED GREEN RED
5.0 mM MgCl2

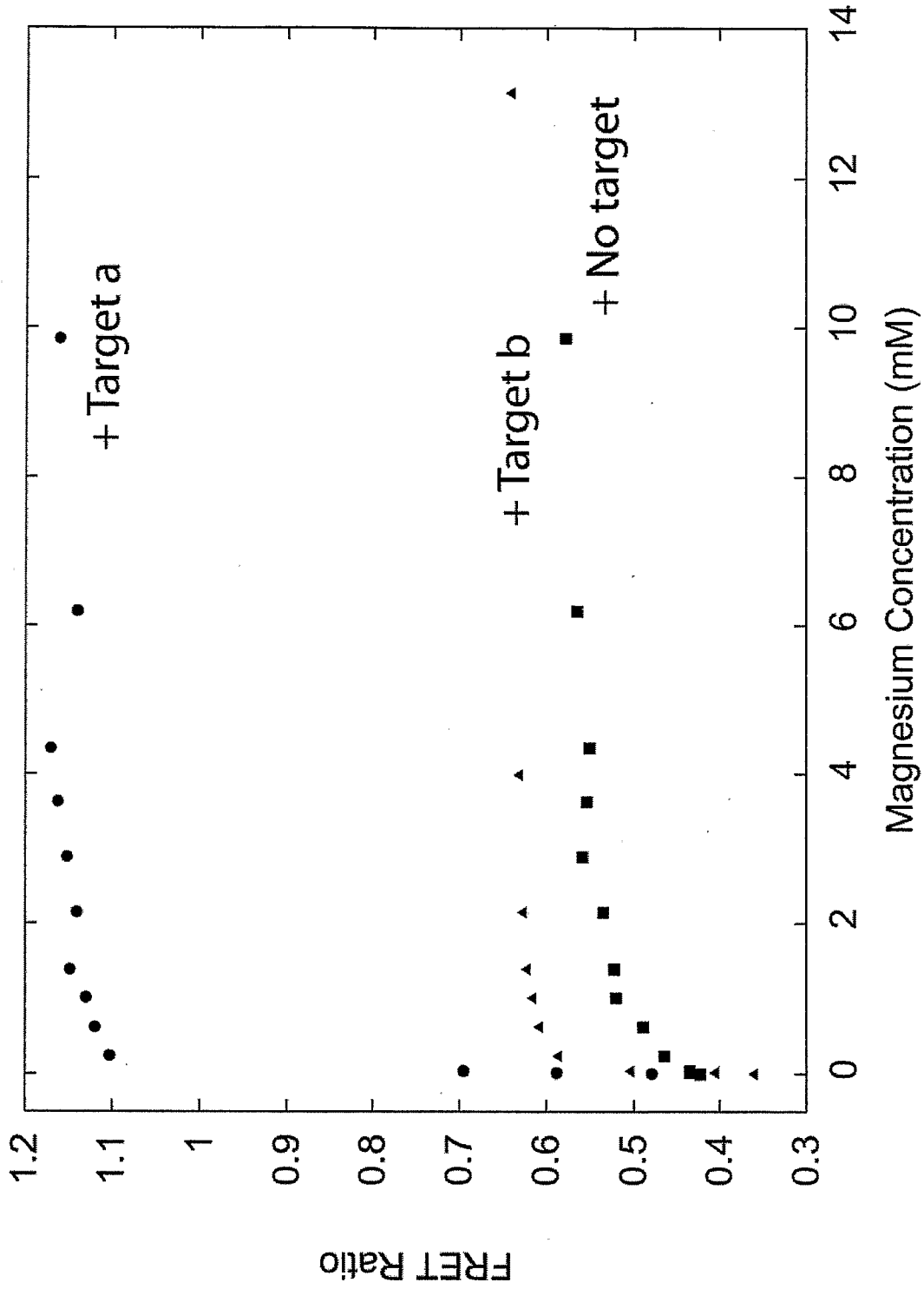

FRET Ratio
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
2
4
6
8
10
12
14
Magnesium Concentration (mM)
+ Target a
+ Target b
+ No target

Figure 16c

Figure 17

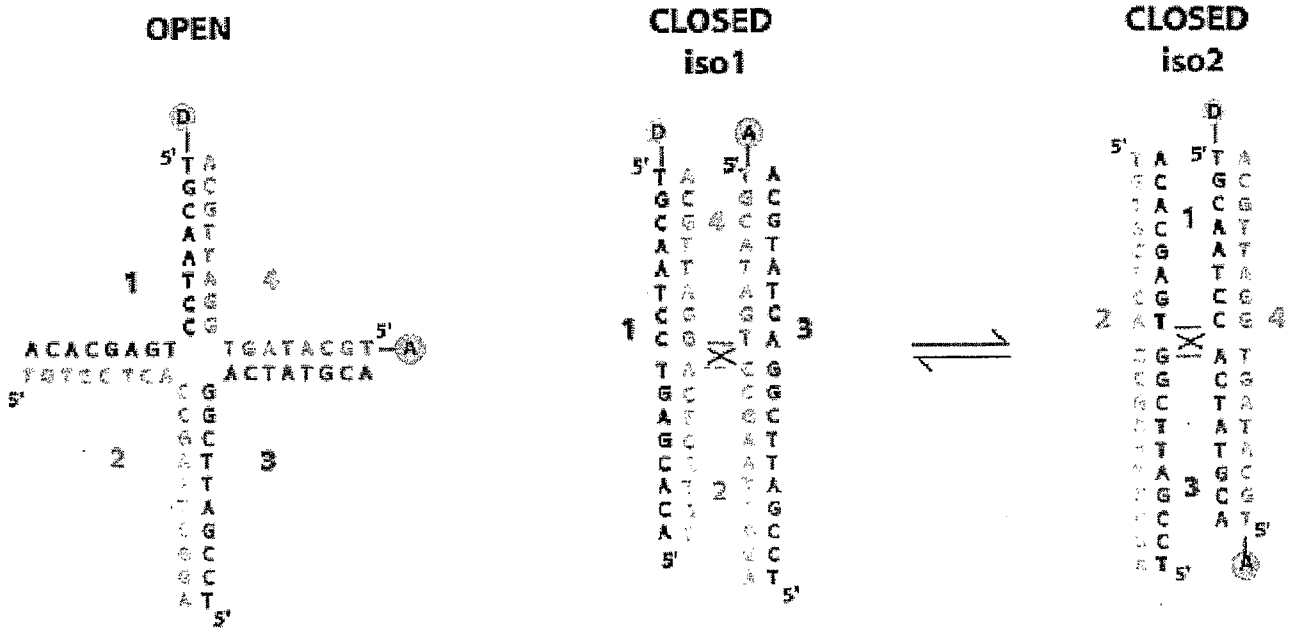


Figure 18

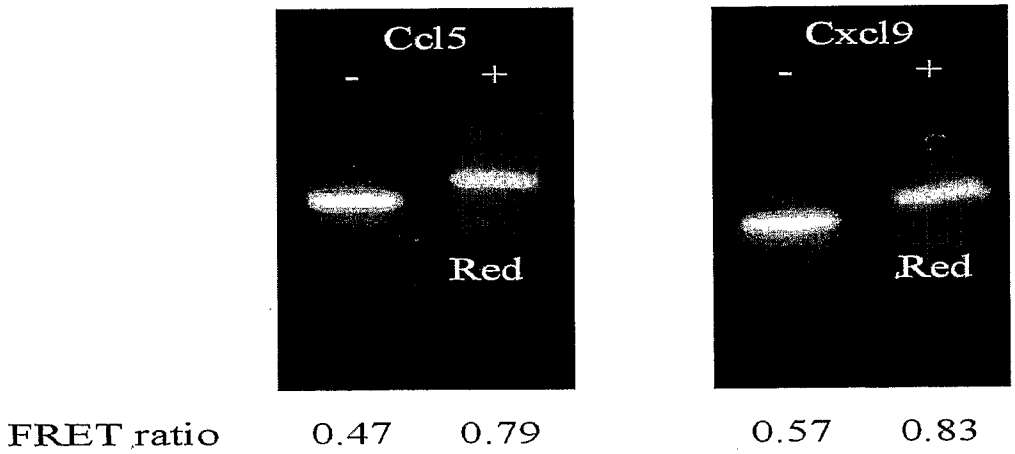
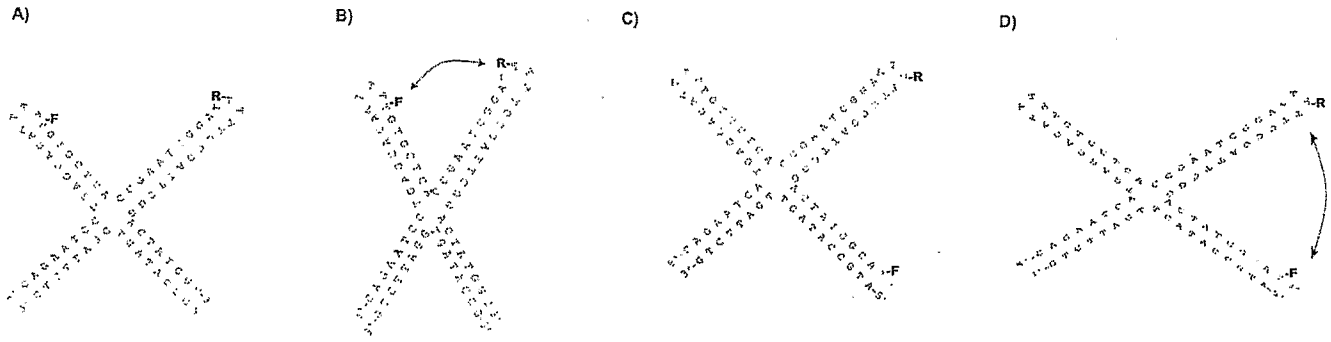


Figure 19



A) HJ A-21311935C, no Mg2+, B) HJ A-2131935C, Mg2+, C) HJ A-2131935A, no Mg2+, D) HJ A-2131935A, Mg2+, F=FAM, R=TAMRA

Figure 20

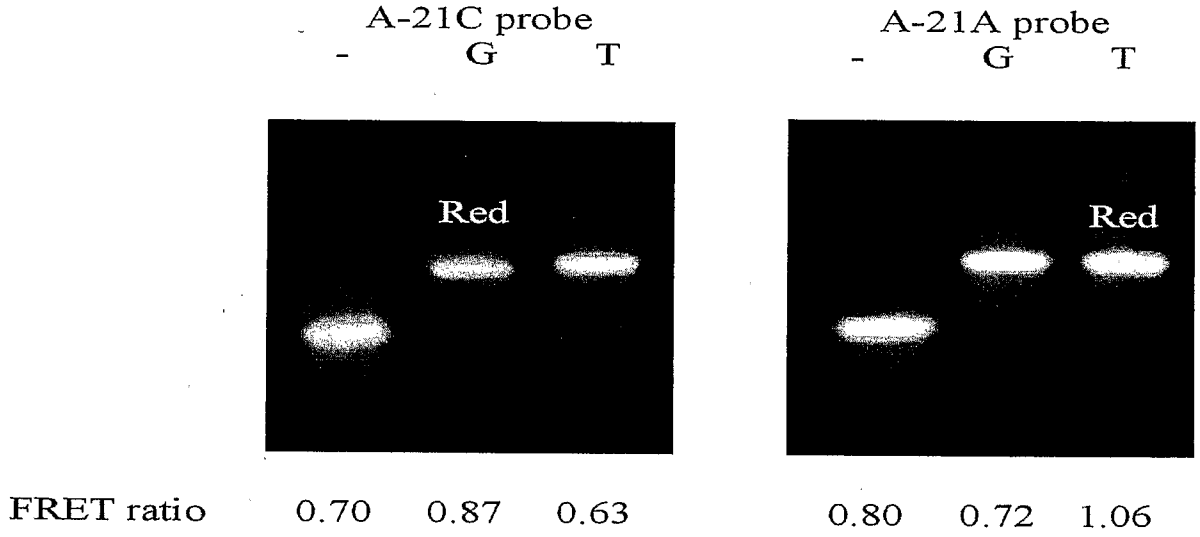


Figure 21

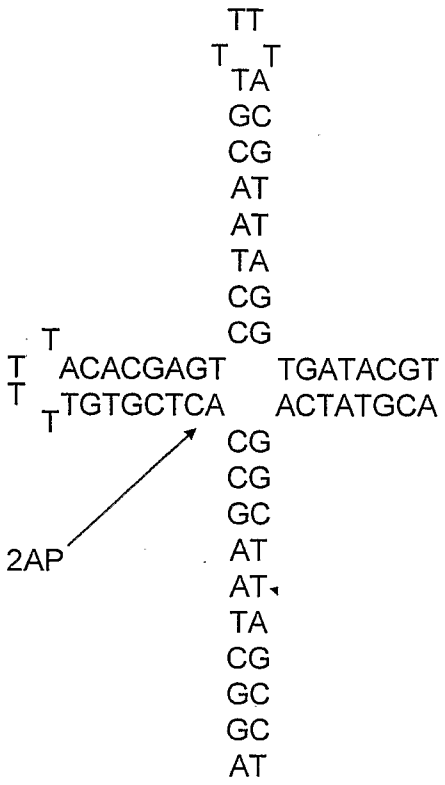


Figure 22

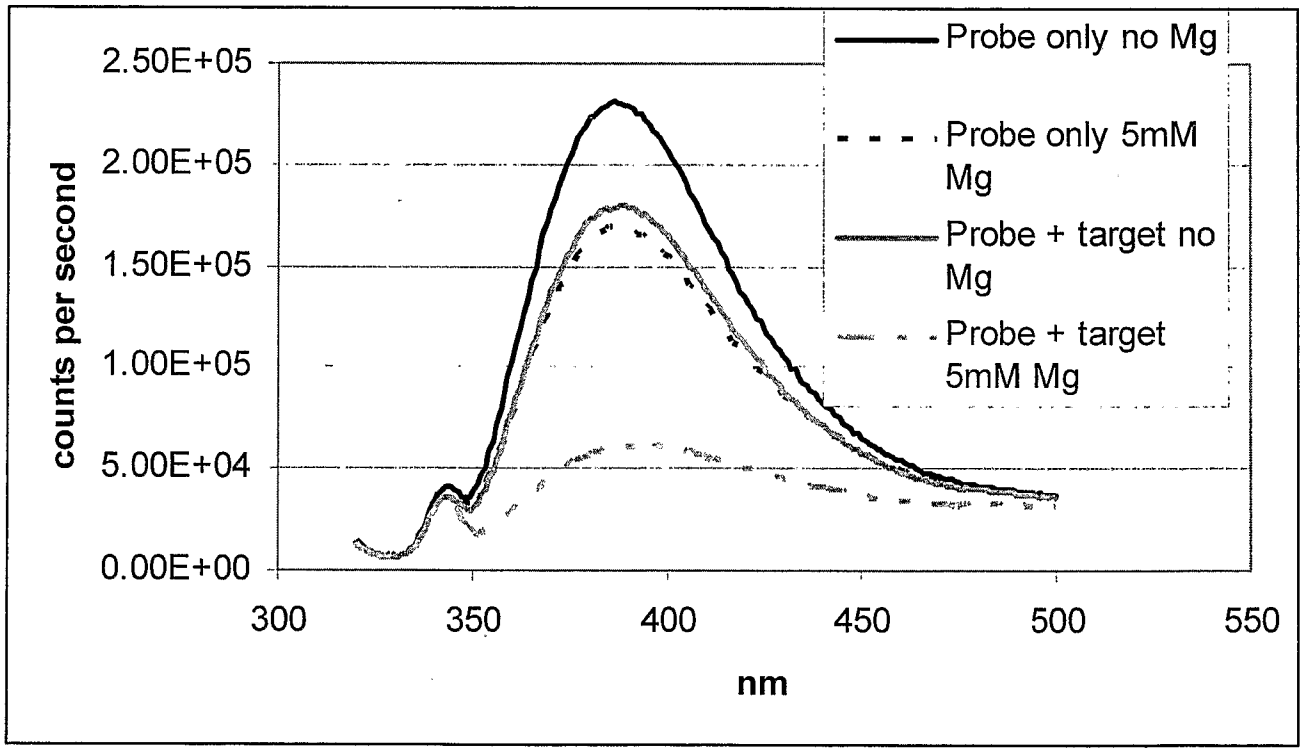
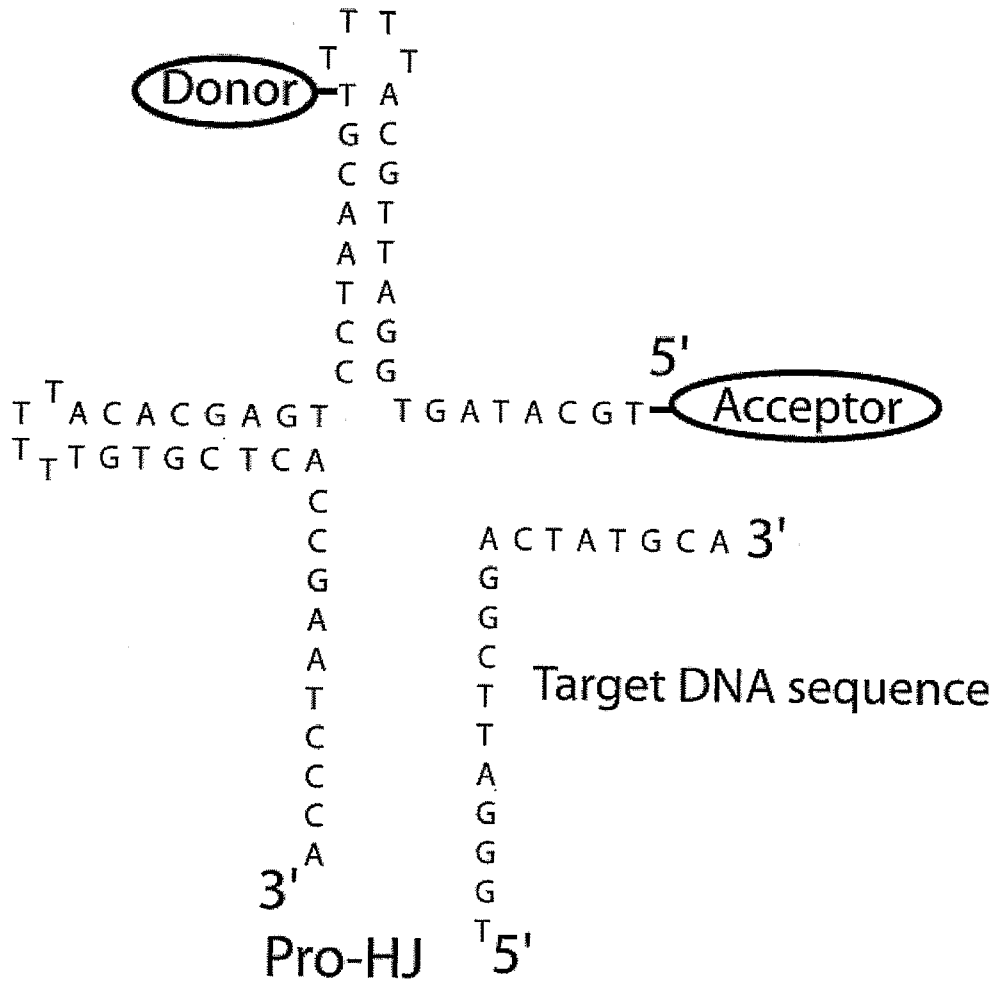


Figure 23



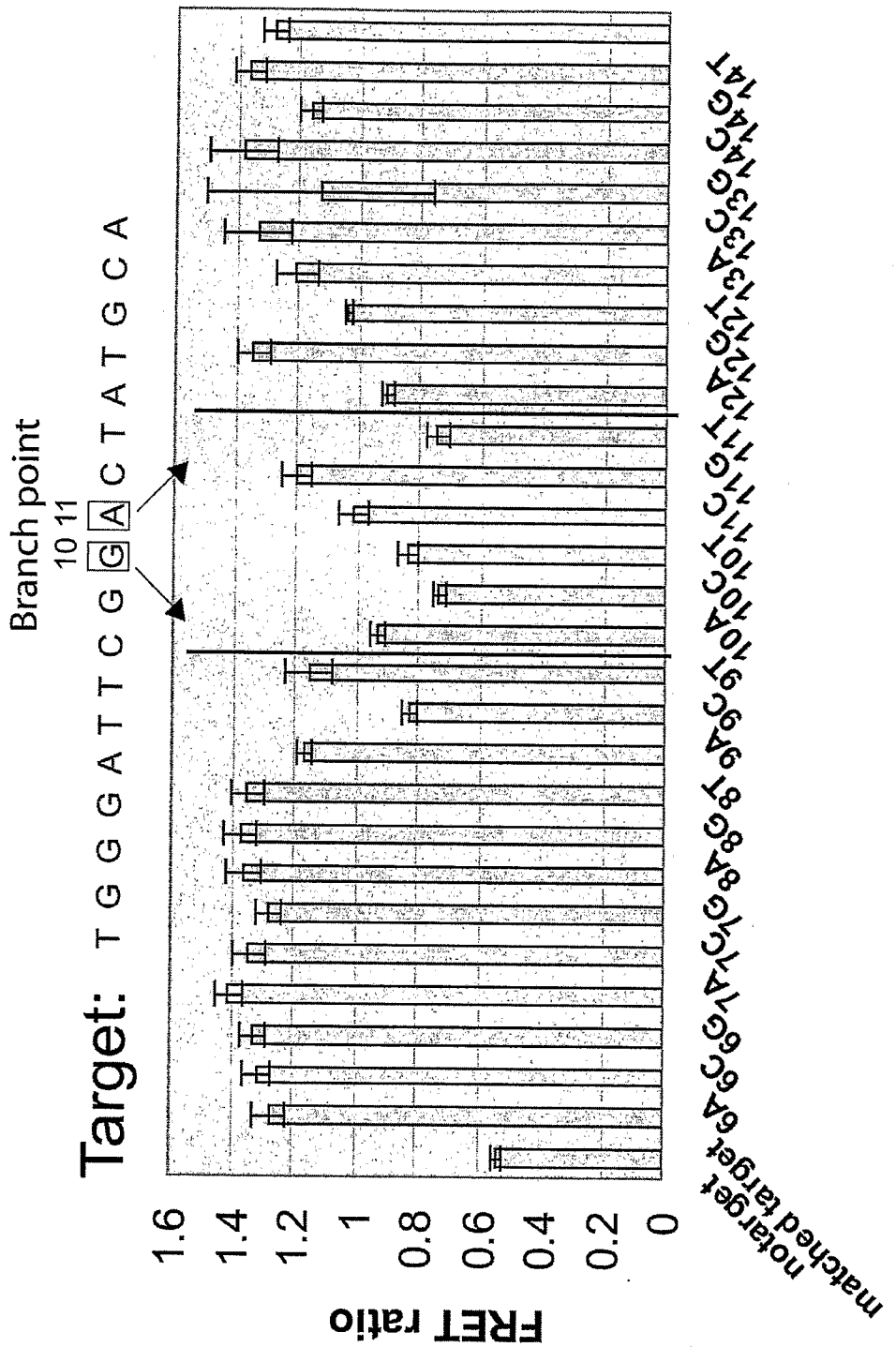
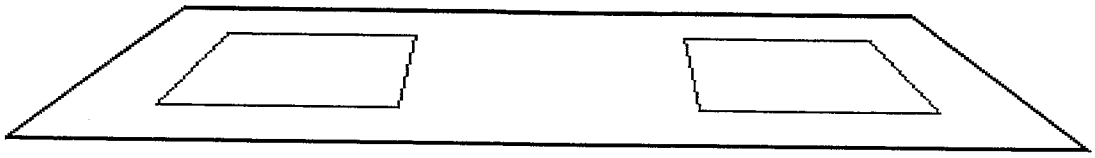


Figure 23 cont.

Figure 24



subarray 1 subarray 2

Figure 25

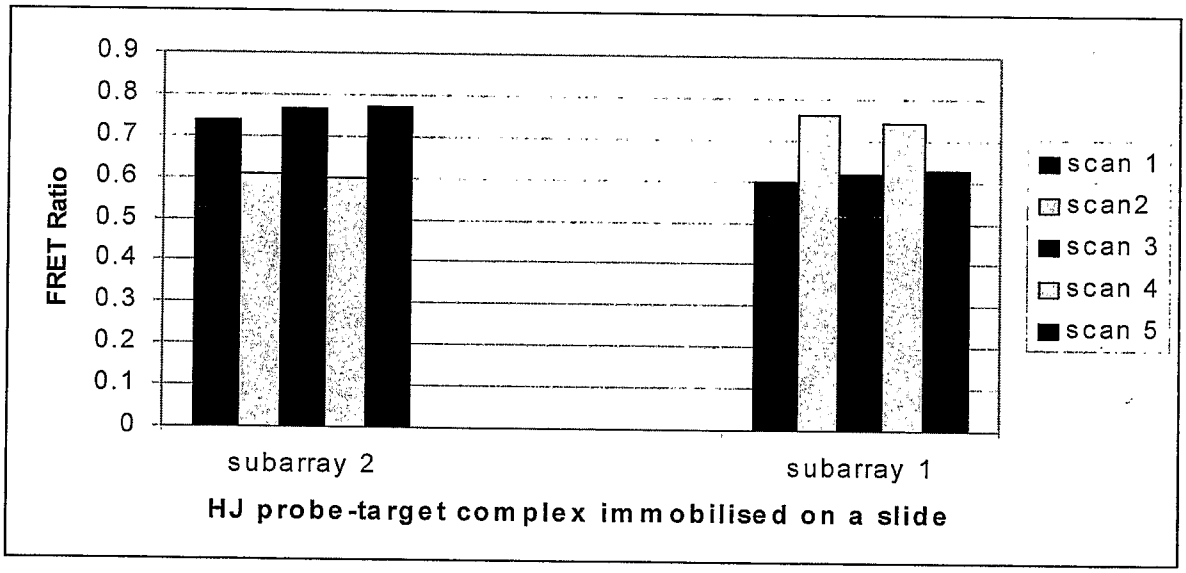


Figure 26

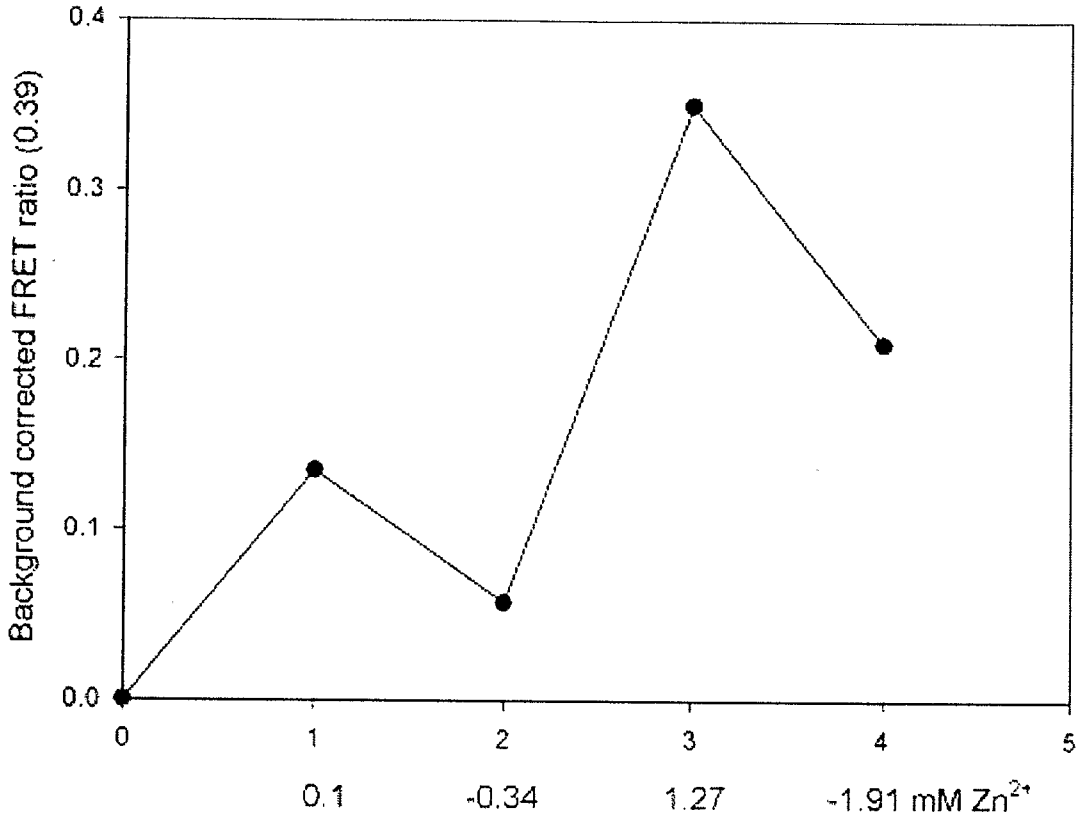
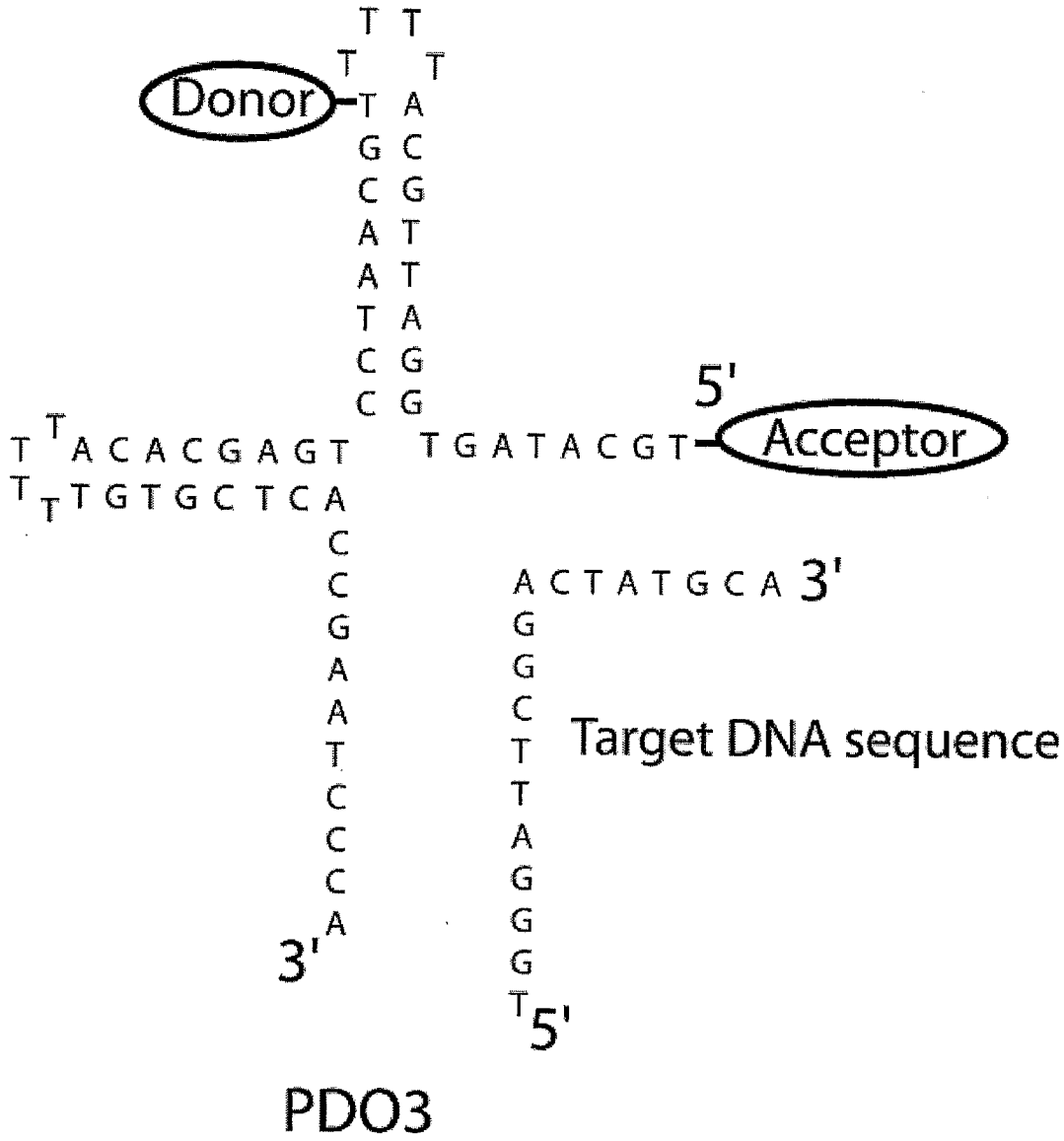

Background corrected FRET ratio (0.39)
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
0.1
-0.34
1.27
-1.91 mM Zn^{2+}

Figure 27a



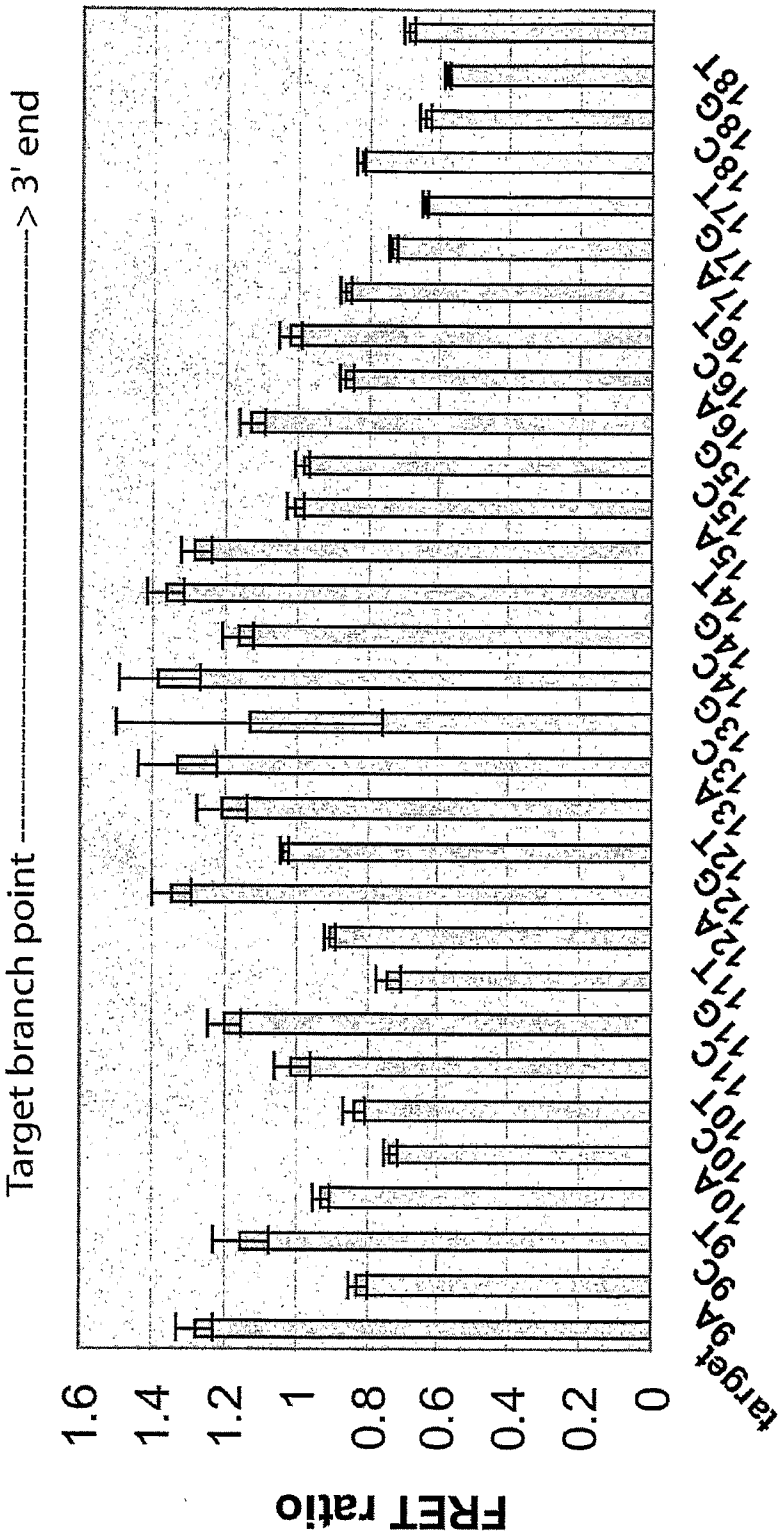
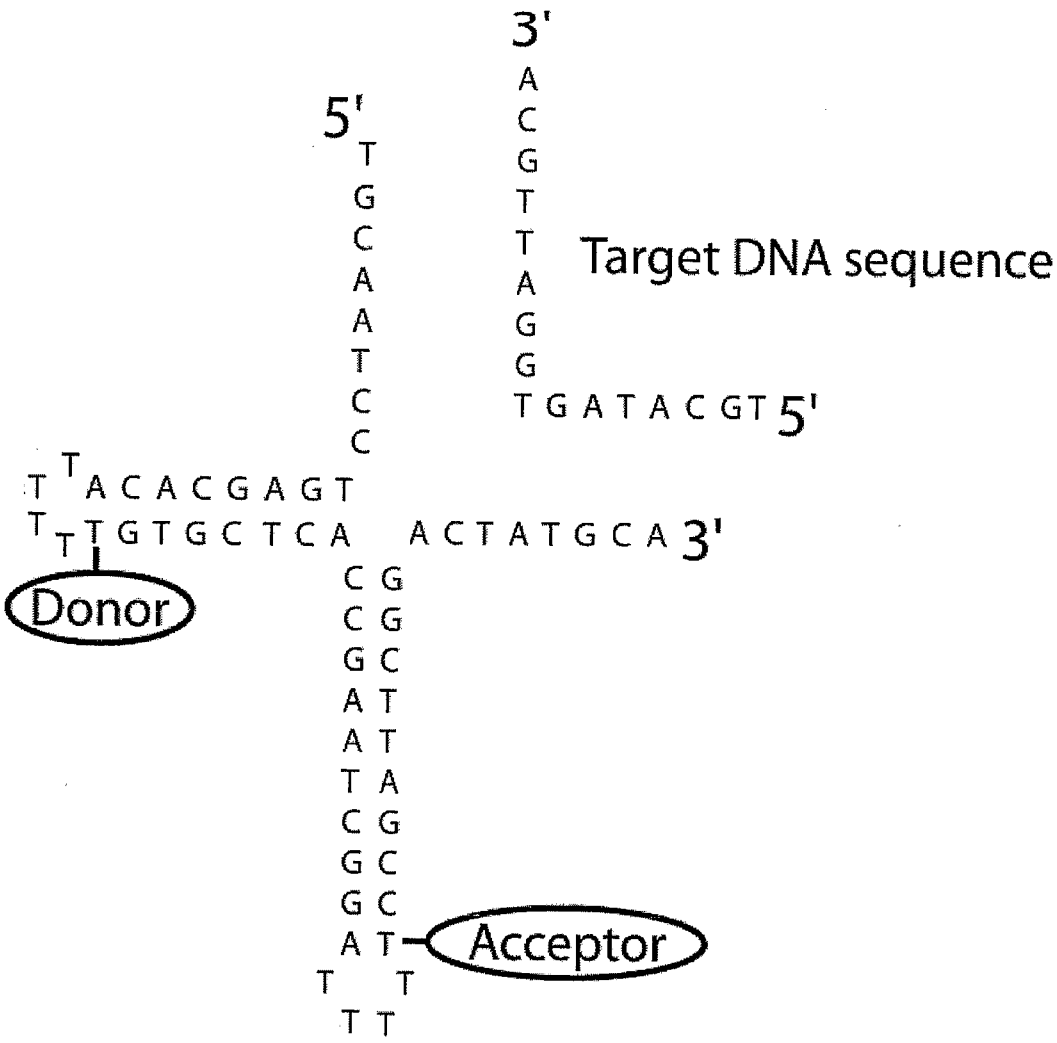

FRET ratio
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Target branch point ----------> 3' end
target 9A 9C 9G 9T 10A 10C 10G 10T 11A 11C 11G 11T 12A 12C 12G 12T 13A 13C 13G 13T 14A 14C 14G 14T 15A 15C 15G 15T 16A 16C 16G 16T 17A 17C 17G 17T 18C 18G 18T

Figure 27b

Figure 27c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A
C
G
T
T
A
G
G
T G A T A C G T 5'
Target DNA sequence
5'
T
G
C
A
A
T
C
C
T
T A C A C G A G T
T T G T G C T C A A C T A T G C A 3'
Donor
C G
C G
G C
A T
A T
T A
C G
G C
G C
A T
Acceptor
T T
T T
PD09

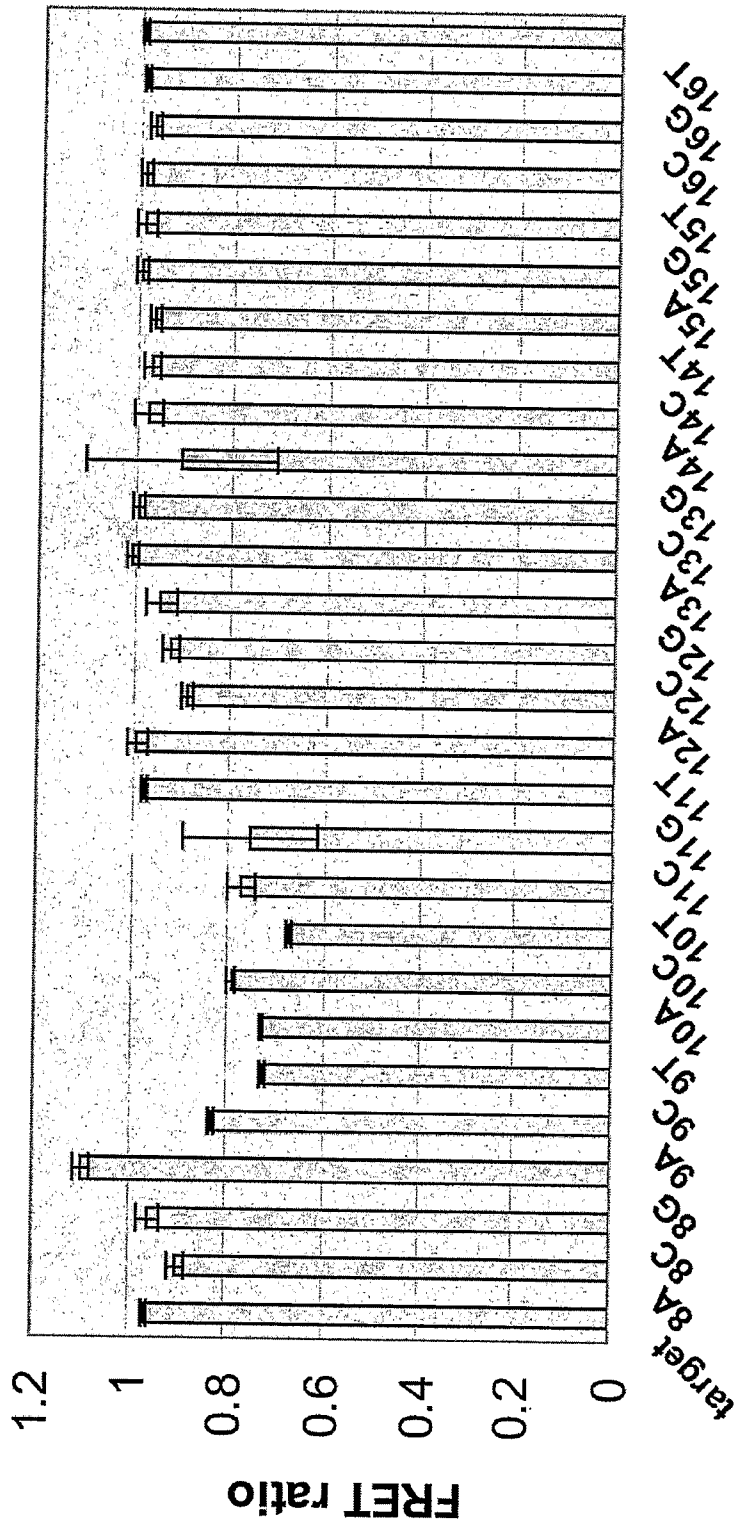

FRET ratio
1.2
1
0.8
0.6
0.4
0.2
0
Target branch point ----------> 3' end
target 8A 8C 8G 9A 9C 9T 10A 10C 10T 11C 11G 11T 12A 12C 12G 13A 13C 13G 14A 14C 14T 15A 15G 15T 16C 16G 16T

Figure 27d

Figure 28

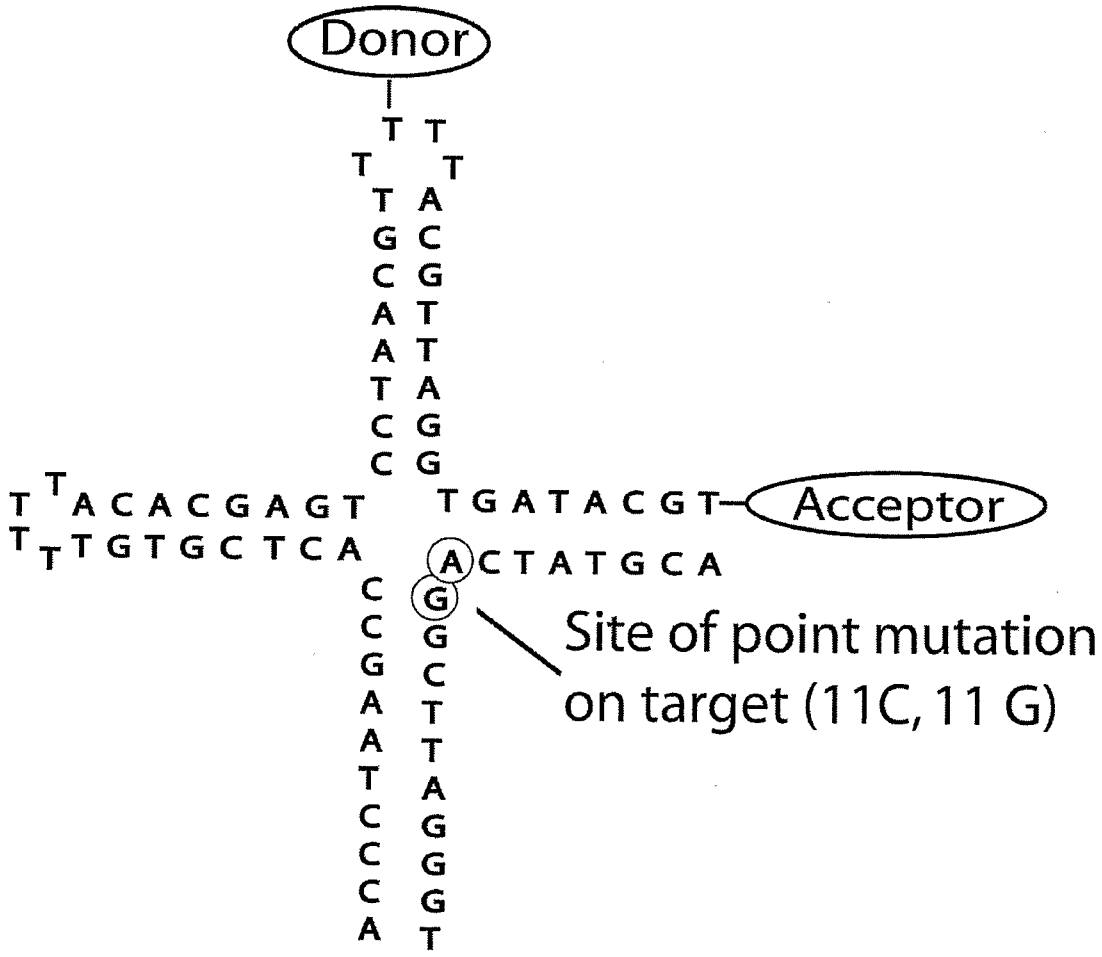

Donor
T T
T T
T A
G C
C G
A T
A T
T A
C G
C G
T T A C A C G A G T
T T T G T G C T C A
T G A T A C G T
Acceptor
A C T A T G C A
C
C
G
A
A
T
C
C
C
A
G
G
C
T
T
A
G
G
G
T
Site of point mutation
on target (11C, 11 G)

Figure 29

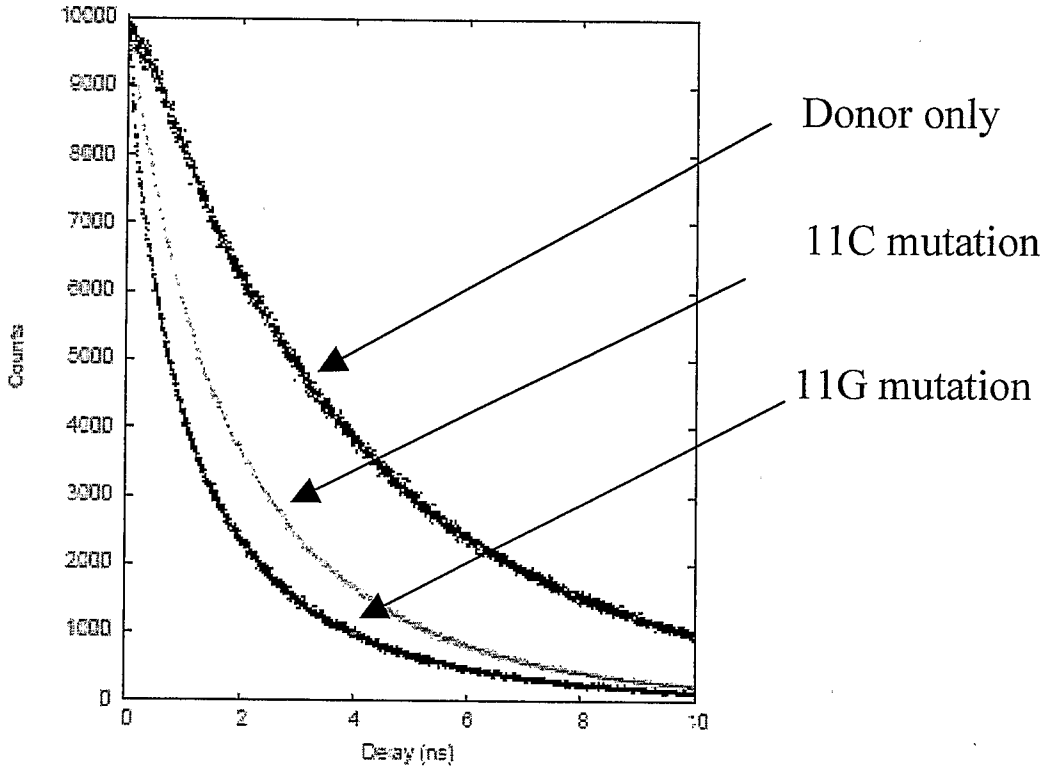

Donor only
11C mutation
11G mutation
Counts
Delay (ns)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
2
4
6
8

Figure 30

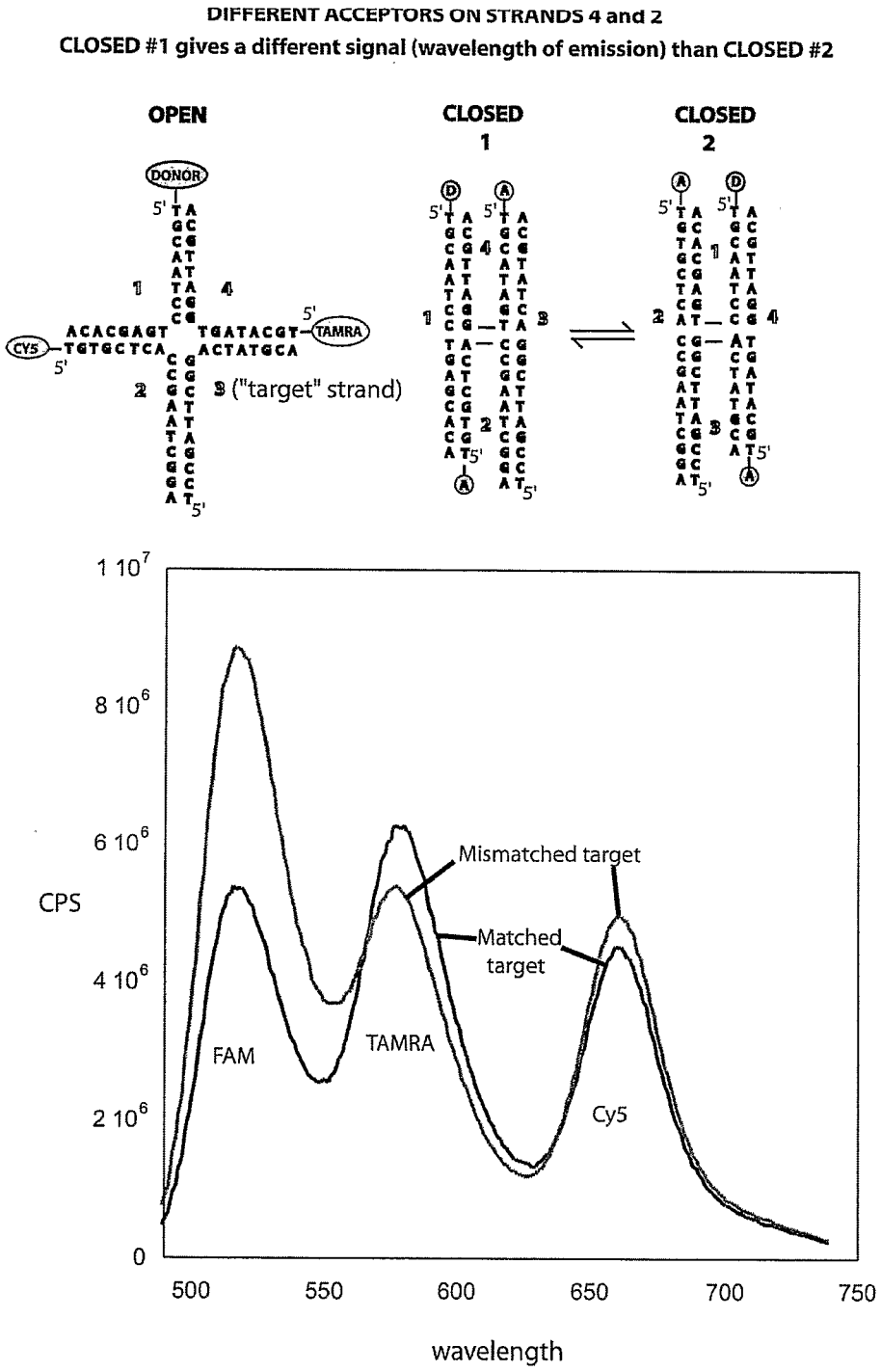
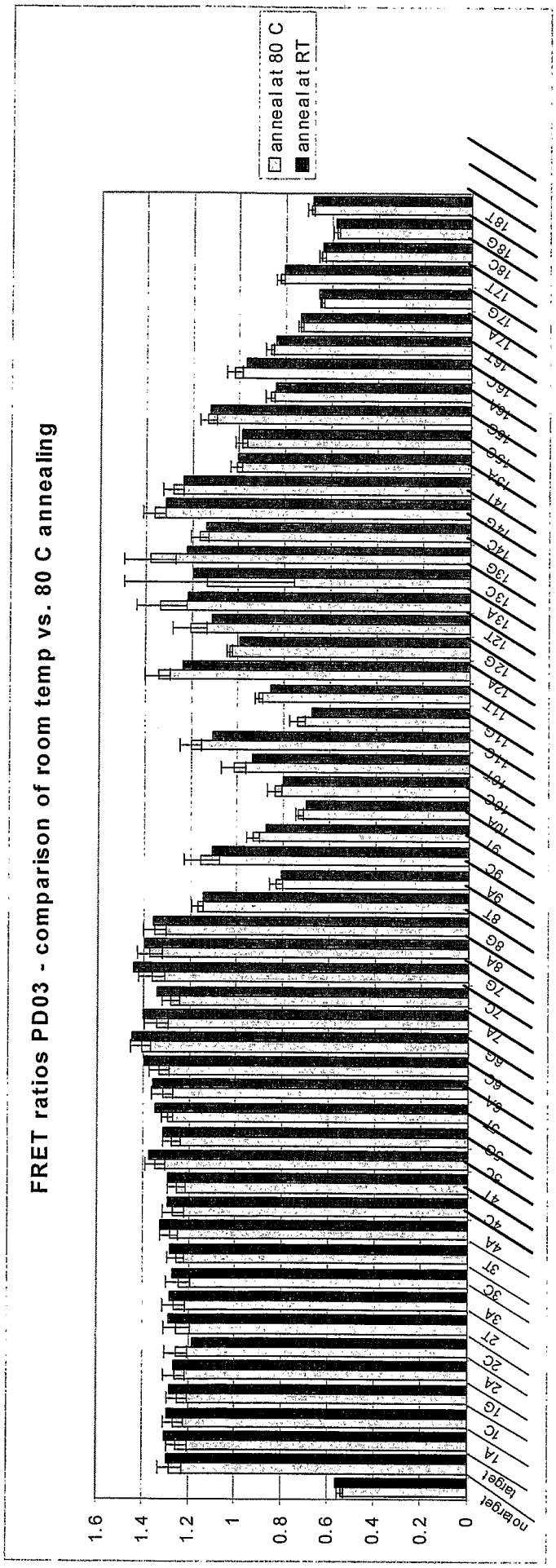

DIFFERENT ACCEPTORS ON STRANDS 4 and 2
CLOSED #1 gives a different signal (wavelength of emission) than CLOSED #2
OPEN
CLOSED 1
CLOSED 2
DONOR
TAMRA
CY5
("target" strand)
1 10^7
8 10^6
6 10^6
4 10^6
2 10^6
0
CPS
Mismatched target
Matched target
FAM
TAMRA
Cy5
500
550
600
650
700
750
wavelength

Figure 31


FRET ratios PD03 - comparison of room temp vs. 80 C annealing
anneal at 80 C
anneal at RT
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1.4
1.2
1
0.8
0.6
0.4
0.2
0
notarget
target
1A
1C
1G
2A
2C
2T
3A
3C
3T
4A
4C
4T
5C
5G
5T
6A
6C
6G
7A
7C
7G
8A
8G
8T
9A
9C
9T
10A
10C
10T
11C
11G
11T
12A
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13A
13C
13G
14C
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15C
15G
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16C
16T
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17G
17T
18C
18G
18T

Figure 32

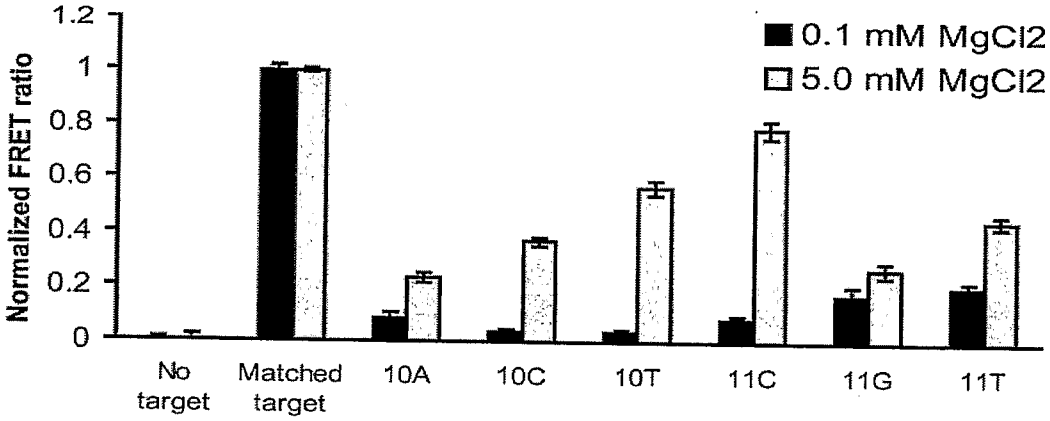
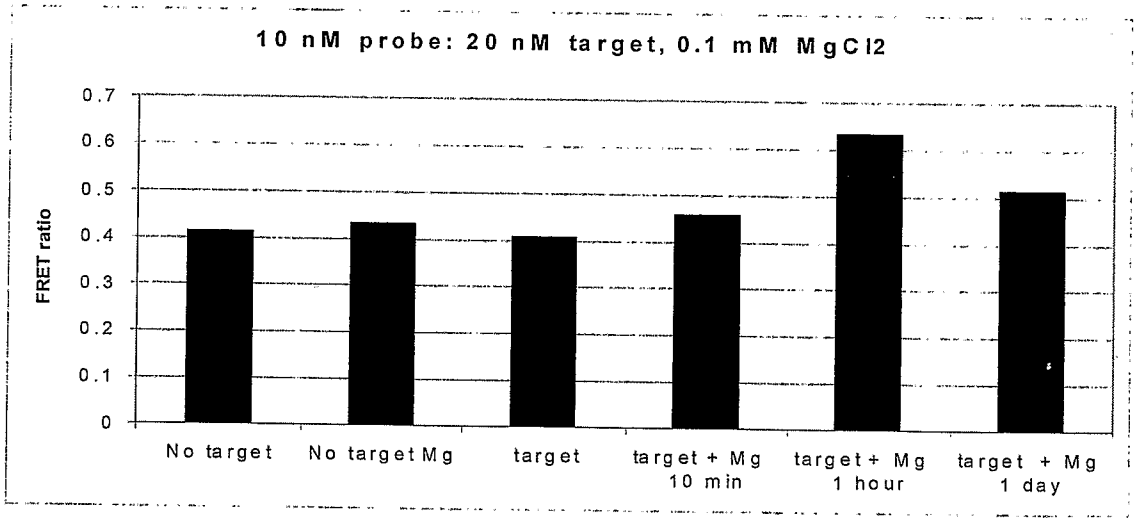


Figure 33



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Buck, Amy J
Dickinson, Paul J
Terry, Jonathan J
Walton, Anthony J
Tereshkova, Elena J
Mountford, Christopher J
Crain, John J
Beattie, John J
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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2006/004161

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 552 931 A1 (GEN PROBE INC [US]) 28 July 1993 (1993-07-28) the whole document figures 21,22B-D	11-31
X	US 2005/014163 A1 (DONG FANG [US] ET AL) 20 January 2005 (2005-01-20) the whole document paragraph [0197]; figures 39,40	11-31

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
19 February 2007	08/03/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Pinta, Violaine

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MCKINNEY S A ET AL: "Structural dynamics of individual Holliday junctions" NATURE STRUCTURAL BIOLOGY, NEW YORK, NY, US, vol. 10, no. 2, 23 December 2002 (2002-12-23), pages 93-97, XP002294545 ISSN: 1072-8368 the whole document page 96, left-hand column	11-31
A	WO 2004/099767 A2 (UNIVERSTY COURT OF THE UNIVERS [GB]; CRAIN JASON [GB]; WALTON ANTHONY) 18 November 2004 (2004-11-18) cited in the application the whole document	
A	LIU JIA ET AL: "Electrostatic interactions and the folding of the four-way DNA junction: analysis by selective methyl phosphonate substitution." JOURNAL OF MOLECULAR BIOLOGY 29 OCT 2004, vol. 343, no. 4, 29 October 2004 (2004-10-29), pages 851-864, XP004599001 ISSN: 0022-2836 cited in the application the whole document	
A	BACKSTROM S ET AL: "The RUNX1 Runt Domain at 1.25A Resolution: A Structural Switch and Specifically Bound Chloride Ions Modulate DNA Binding" JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 322, no. 2, 13 September 2002 (2002-09-13), pages 259-272, XP004449744 ISSN: 0022-2836 cited in the application the whole document	
A	ROSENGARTH A ET AL: "A Calcium-driven Conformational Switch of the N-terminal and Core Domains of Annexin A1" JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 326, no. 5, 7 March 2003 (2003-03-07), pages 1317-1325, XP004454106 ISSN: 0022-2836 cited in the application the whole document	

-/--

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2006/004161

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	MOUNT A R ET AL: "The stability and characteristics of a DNA Holliday junction switch." BIOPHYSICAL CHEMISTRY 1 DEC 2006, vol. 124, no. 3, 1 December 2006 (2006-12-01), pages 214-221, XP002420133 ISSN: 0301-4622 the whole document	
A	US 2005/205434 A1 (SEN DIPANKAR [US] ET AL) 22 September 2005 (2005-09-22) the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2006/004161

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0552931 A1	28-07-1993	AU 665062 B2	14-12-1995
		AU 3586693 A	01-09-1993
		CA 2128530 A1	05-08-1993
		DE 69328699 D1	29-06-2000
		DE 69328699 T2	07-09-2000
		JP 3415150 B2	09-06-2003
		JP 7503139 T	06-04-1995
		WO 9315102 A1	05-08-1993
		US 5451503 A	19-09-1995
		US 5424413 A	13-06-1995
US 2005014163 A1	20-01-2005	NONE	
WO 2004099767 A2	18-11-2004	CA 2524655 A1	18-11-2004
		CN 1816920 A	09-08-2006
		EP 1620719 A2	01-02-2006
		GB 2417777 A	08-03-2006
		JP 2006525662 T	09-11-2006
US 2005205434 A1	22-09-2005	NONE	